Modern Techniques for Pathogen Detection

Edited by Jürgen Popp
and Michael Bauer

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Contents

Preface *XI*
List of Contributors *XV*

1 **Unmet Medical Needs in Life-Threatening Infections – Caring for the Critically Ill** *1*
Michael Bauer, Andreas Kortgen, and Mervyn Singer
1.1 Life Threatening Infections and Sepsis – Defining the Problem *1*
1.2 Sepsis as a "Hidden Healthcare Disaster" *3*
1.3 Microorganisms and Types of Infection Triggering Sepsis *4*
1.4 Emerging Problems Related to Resistance in Bacterial Infections *5*
1.5 The Role of Fungi and Viruses *5*
1.6 The Need for New Approaches in Diagnostics of Life-Threatening Infection and Sepsis *7*
1.7 Rapid and Sensitive Culture-Independent Strategies to Identify Blood Stream Infection *8*
1.8 Beyond Infection – Profiling the Immune Response of the Septic Host *10*
1.9 Host Factors Contributing to Pathogenesis of Sepsis *11*
References *13*

2 **Identification Methods – An Overview** *19*
Oliwia Makarewicz, Claudia Stein, Wolfgang Pfister, Bettina Löffler, and Mathias Pletz
2.1 Taxonomy of Pathogenic Organisms *19*
2.2 Microscopic Methods *20*
2.2.1 Stains *20*
2.2.2 Autofluorescence *29*
2.2.3 Immunofluorescence *30*
2.2.4 *In Situ* Hybridization *30*
2.2.5 Dark-Field Microscopy *31*
2.3 Culture-Based Methods *31*
2.3.1 Blood Culture *32*

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2.3.2 Automated Differentiation Systems *33*
2.3.3 API System *35*
2.3.4 Selective Agar *36*
2.3.5 Susceptibility Testing *37*
2.3.6 Other Methods *38*
2.4 Nucleic Acid-Based Techniques *38*
2.4.1 PCR *39*
2.4.1.1 Real-Time PCR *39*
2.4.1.2 Multiplex-PCR *40*
2.4.1.3 RAPD and rep-PCR *40*
2.4.1.4 Microarrays *40*
2.4.1.5 RFLP *41*
2.4.2 Sequencing *41*
2.4.2.1 Ribosomal RNA Genes *41*
2.4.2.2 MLST *42*
2.4.2.3 NG Sequencing *42*
2.5 Serology *42*
2.5.1 Antigen–Antibody-Based Methods *44*
2.5.1.1 Agglutination *44*
2.5.1.2 Immunodiffusion *45*
2.5.1.3 Quantitative Immunoassays *45*
2.5.2 Automated Immunoassays *46*
2.5.3 Applications of Serological Test *47*
2.5.3.1 Types of Human Antibodies and What They Indicate? *47*
2.5.3.2 Validity of Antibodies against Opportunistic Species *47*
2.5.3.3 Specificity of Fungal Antigens *48*
2.5.3.4 Specific Urine Antigen Tests *48*
2.5.4 Biomarkers *48*
2.6 Conclusions and Perspectives *49*
References *50*

3 Nucleic Acid Amplification Techniques *55*
Marc Lehmann and Roland P.H. Schmitz
3.1 Introduction *55*
3.2 The Basic PCR Protocol *56*
3.3 Primer Design *60*
3.3.1 Specific versus Broad-Range Primers and the Fate of False Targeting *63*
3.4 Modified End-Point PCR Protocols *66*
3.5 Non-PCR NAT: Isothermal Amplification Protocols *70*
3.6 Quantitative PCR *73*
3.6.1 Quantification in qPCR *77*
3.6.2 Melting Curve Analysis (MCA) *78*
3.7 Controls, Probing, and General Aspects of Result Interpretation *78*
3.7.1 Strategies in Amplicon Verification *80*

3.7.1.1 (DNA) Microarray *80*
3.7.1.2 Flow Cytometry *81*
3.7.1.3 Mass Spectrometry *81*
3.7.2 PCR Inhibitors *82*
3.8 Preanalytics *83*
3.8.1 Sample Volume *83*
3.8.2 Cell Lysis *84*
3.8.3 Nucleic Acid Isolation and Preparation *85*
3.9 Fields of PCR Application *86*
3.9.1 Sequencing *86*
3.9.1.1 Pyrosequencing *87*
3.9.2 Typing and Epidemiology *88*
3.9.3 Pathogen Detection in Complex Clinical Samples *89*
3.9.4 General Aspects of Result Interpretation *90*
3.9.4.1 Sensitivity and Specificity *90*
3.9.4.2 NAT versus Culture *92*
3.9.4.3 The Fate of Antibiotic Resistances Detection *93*
3.10 Microbial Trace Detection in BSI, Ascites, and Synovial Fluids *94*
3.10.1 Grown Blood Culture Media as Samples *97*
3.11 Upcoming Routine Solutions – Dawn of Assay Automation *98*
3.12 Conclusion and Perspective *100*
Acknowledgment *101*
References *102*

4 DNA Microarrays for Pathogen Detection *113*
Holger Schulze, Maya Rubtsova, and Till T. Bachmann
4.1 Introduction *113*
4.2 DNA Microarrays for the Detection of Bacterial Pathogens *114*
4.2.1 Major Bacterial Diseases *114*
4.2.1.1 Blood Stream Infections/Sepsis *114*
4.2.1.2 Pneumonia *122*
4.2.1.3 Infectious Diarrhea *123*
4.3 Antibiotic Resistance Detection *155*
4.3.1 Gram-Negative Bacteria *165*
4.3.2 Gram-Positive Bacteria *170*
4.3.2.1 *Staphylococcus aureus* *170*
4.3.2.2 Mycobacteria *171*
4.4 DNA Microarrays for Virus Diagnostics *173*
4.4.1 Human Papillomaviruses *177*
4.4.1.1 Commercial HPV Tests *178*
4.4.2 Respiratory Viruses *180*
4.4.3 Enteric Viruses *182*
4.4.4 Hepatitis Viruses *182*
4.5 DNA Microarrays for Detection of Fungal Pathogens *184*
4.6 DNA Microarrays for Parasite Diagnostics *200*

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4.6.1 Malaria *200*
4.6.2 Waterborne Parasites *201*
4.6.3 Other Parasites *203*
4.7 Conclusion an Outlook *207*
References *207*

5 MALDI-ToF *221*
Stefan Zimmermann
5.1 Introduction *221*
5.2 MALDI-ToF Technology *222*
5.3 Bacterial Identification Using Mass Spectrometry *225*
5.4 Culture-Independent Rapid Identification of Clinical Pathogens *234*
5.5 Antibiotic Susceptibility Testing Using Mass Spectrometry *239*
References *244*

6 IR and Raman Spectroscopy for Pathogen Detection *253*
Ute Münchberg, Sandra Kloß, Dragana Kusić, Susann Meisel, Ralf Heinke, Stephan Stöckel, Petra Rösch, and Jürgen Popp
6.1 Introduction *253*
6.2 Bulk Analysis *256*
6.2.1 IR and NIR Absorption Spectroscopy *256*
6.2.2 Raman Spectroscopy with Excitation in the NIR Region *260*
6.2.3 Resonance Raman Spectroscopy *263*
6.3 Excitation with Visible Wavelengths *264*
6.4 UV-Resonance Raman Spectroscopy *266*
6.4.1 Surface-Enhanced Raman Spectroscopy *270*
6.4.1.1 The SERS Effect *270*
6.4.1.2 Analysis of Bulk Bacteria Samples with SERS *272*
6.5 Single-Cell Analyses *276*
6.5.1 Micro-Raman Spectroscopy with Excitation in the Visible or NIR Region *276*
6.5.2 Surface-Enhanced Raman Spectroscopy on Single Cells *284*
6.6 Conclusion and Outlook *287*
References *288*

7 FISH *295*
Graeme N. Forrest, Jwan Mohammadi, and Shahrzad Mohammadi
7.1 Introduction *295*
7.2 FISH Techniques *295*
7.2.1 PNA FISH *296*
7.2.1.1 Original PNA FISH Platform *296*
7.2.1.2 PNA FISH Flow Cytometry *297*
7.2.1.3 Beacon-Based FISH (bbFISH) *298*
7.3 Clinical Implications of PNA FISH *299*

7.3.1 Gram-Positive Organisms *299*
7.3.1.1 Staphylococci *299*
7.3.1.2 Enterococci *302*
7.3.2 Gram-Negative Probes *303*
7.3.2.1 Candida *305*
7.3.2.2 Beta Hemolytic Streptococci *309*
7.3.2.3 Mycobacteria *310*
7.3.2.4 Helicobacter *p*ylori *310*
7.3.2.5 Other Bacteria *311*
7.3.2.6 Parasites *311*
7.4 Summary and Outlook *311*
References *312*

8 Conclusions *319*
Sibyll Pollok, Karina Weber, Michael Bauer, and Jürgen Popp

Index *323*

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Preface

This book gives a comprehensive overview of the frontline research in pathogen detection. Infectious diseases caused by bacterial, viral, or fungal pathogens are one of the leading causes of death worldwide. In order to find the optimal treatment regime for the highly heterogeneous group of patients and to significantly reduce costs in medical care, novel tools that characterize the pathogenic microbes and the host-specific immune response are highly desired. Information about the phenotype and genotype of the germs help to accelerate quick and effective interventions, for example, the treatment of a sepsis patient with the right medication. Especially, within this context, the terms personalized medicine and pharmacogenomics, which define a stratified treatment for each individual patient, are of increasing importance. Due to a high variety in the responses of a patient panel to similar drug treatment regimes, which are caused by genetic and/or physiological variations among patients, the therapy has to be adapted to achieve highest efficiency and lowest side effects.

A plethora of analytical methods such as spanning microbiology, biochemistry, molecular biology, immunology, and biophysics are commonly applied for the detection of pathogens. *Modern Techniques for Pathogen Detection* informs in detail about various cutting-edge tools for pathogen identification. International scientists of the respective areas highlight recent emerging methods as well as improvements of conventional assays within individual chapters.

The management of life-threatening infections, especially sepsis, is covered, due to its importance, in a separate chapter of the book (Chapter 1). In the case of a sepsis suspicion, pathogen detection and administration of the appropriate drug combination should be performed as soon as possible. We stand at the beginning of a more personalized approach toward sepsis care. The novel developed analytical tools can facilitate an early and precise diagnosis of the pathogenic causatives of the severe infection concomitant, with profiling of their genetic status in terms of resistance to a certain antimicrobial therapy and finally also the monitoring of the treatment response.

Conventional techniques, involving microscopic or culture-based identification, enable the reliable detection of bacteria at low concentrations (Chapter 2). The morphological rating of bacteria from human patient samples combining microscopy with several classical or immunological staining techniques is an

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inherent part of everyday clinical practice. Beyond that, the gold standard in routine pathogen diagnostics is based on culture methods, which use selective growth media in combination with a multitude of biochemical tests. These tests are inexpensive and provide qualitative and quantitative information about the investigated pathogens. Culture-based techniques usually require an overnight incubation, thus the analysis time is extended to at least 1 day. In addition, the culture-based approach relies on the presence of culturable cells. Therefore, only the implementation of testing results from other assays provides the possibility to evaluate the pathogenic flora in case of non-culturable or slow-growing organisms.

In the last decades, molecular assays improved pathogen detection, leading to higher sensitivity, selectivity, and a significantly reduced sample-to-answer time. Nucleic acid-based techniques are discussed in detail in the relevant chapters (Chapters 3, 4, and 7). Great efforts were made to enable a simultaneous screening of several pathogens by means of multiplex nucleic acid amplification or microarray platforms. The trend to integrate automation in the molecular assay workflow has led to sample-to-answer times of several hours, depending on the employed diagnostic technique. Nevertheless, the results of PCR and microarray approaches should be critically rated in the clinical and microbiological context of the patients. An elegant assay is the direct specification of pathogens within a blood culture by means of fluorescence *in situ* hybridization (FISH). This approach is a follow-up staining method after the initial identification of Gram-positive or Gram-negative bacteria. A Quick-FISH testing platform can minimize the sample-to-answer time starting from Gram stain to 30 min. Of great importance is the fact that the inclusion of nucleic acid-based techniques into clinical routine depends on both the total hands-on-time and operator-friendly analysis platforms.

Furthermore, modern analytical methods, such as MALDI-TOF mass spectrometry, are discussed as a routine clinical microbiology laboratory for identification of infectious agents (Chapter 5). This fast evolving and increasingly applied technique allows the rapid and accurate identification of microorganism as well as antibiotic resistance markers based on specific biomarker signatures. Within this context, this pattern-matching process offers the balance between the time-consuming culture-based techniques and highly accurate nucleic acid-based techniques. Very promising tools for pathogen profiling are IR and Raman spectroscopic techniques, which are therefore also introduced within the book (Chapter 6). Their main advantage is given by the possibility of probing bacteria at single-cell level without any prior cultivation steps. IR and Raman spectroscopic techniques are successfully applied to identify bacteria by matching experimental molecular fingerprint information of single bacteria with reference members of the same biological species in preformed spectral databases by chemometrics. This pattern-matching approach is described for the direct classification and identification of infectious agents.

In summary, *Modern Techniques for Pathogen Detection* highlights state-of-the-art knowledge, emerging trends, and critical advices from experts for pathogen

detection. We highly recommend the book to interdisciplinary working scientists at the nexus of fundamental research and clinical application, which plan to focus their research on further improvements that enable accurate and timely pathogen identification along with the respective antibiotic resistance information. The latter one is definitely the prerequisite to quickly adjust the right medication for a patient who suffers from severe microbial infection. A further crucial challenge for all the applied tools in terms of pathogen detection is the precise distinction of normal colonization and pathogenic invasion.

Last but not least, we would like to thank all the authors for the enjoyable cooperation that contribute to this book. The authors have a designated longtime expertise on their respective scientific area within the fast evolving field of microbial infection detection. Special thanks for their conscientious work, the abundance of patience, and the countless fruitful discussions. We would also thank the dedicated team of the publisher.

Michael Bauer
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Jena, January 2015

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1
Unmet Medical Needs in Life-Threatening Infections – Caring for the Critically Ill

Michael Bauer, Andreas Kortgen, and Mervyn Singer

1.1
Life Threatening Infections and Sepsis – Defining the Problem

The large number of infectious agents, complicated further by many varied pathogen- and host-specific characteristics, results in a broad spectrum of communicable diseases of which both prevention and control are challenging. While many infectious diseases are benign and are primarily treated in the community, severe infections may give rise to an urgent need to control the source of infection, to implement appropriate anti-infective therapy, and to provide supportive care to maintain homeostasis [1].

Under these conditions, the patient outcome from infection is determined not only by the invading pathogen which can be directly toxic and destructive to cells and tissues but also – or even primarily – by the host response. This host response may be inappropriately exaggerated, leading to severe tissue injury. Here, the effector molecules of immune cells, such as oxygen free-radicals and nitric oxide, cannot discriminate between microbial targets and host tissue [2]. Indeed, a novel concept has been proposed to describe the development of organ failure, that is, severe sepsis, as a disturbed "disease tolerance" where the eventual development of organ dysfunction is considered an inability to establish an appropriate equilibrium between direct pathogen damage and the ensuing host response (Figure 1.1) [3]. Patients with an uncontrolled focus of infection or an exuberant host response are particularly prone to develop organ dysfunction requiring care in a specialized "intensive care unit (ICU)." Such patients are referred to as *septic* (Figure 1.2).

Sepsis is defined and diagnosed by nonspecific alterations in temperature, heart and respiratory rate, and white cell count secondary to infection (Table 1.1) [4]. Unfortunately, in current clinical practice, neither the causative pathogen nor the specific cellular processes underlying deterioration of organ function that would be amenable to specific therapeutic intervention can be assessed in a way that would allow tailoring of anti-infective or immunomodulatory therapies to specific patient needs. This is particularly relevant given the pressing need to respond within the first few "golden" hours. These shortcomings regarding "point-of-care" diagnostics are in sharp contrast to the burgeoning development of sophisticated

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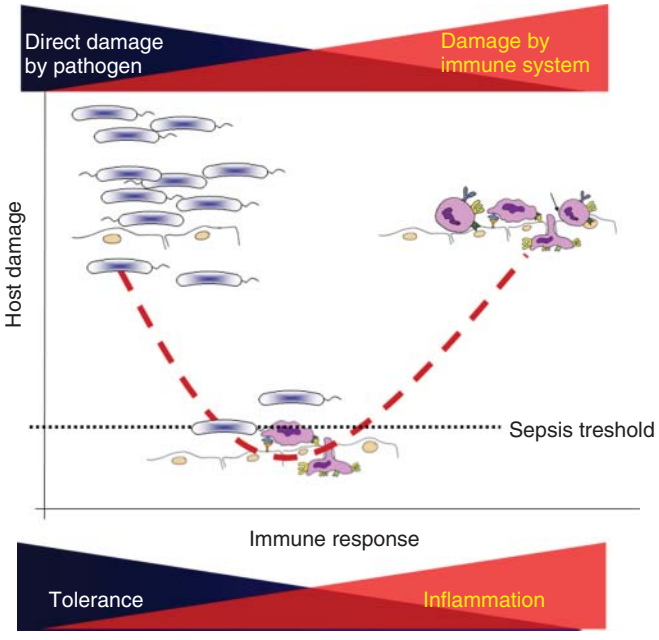

Figure 1.1 Evolving concepts of sepsis as a "host defense failure disease." The host response to invading pathogens requires a cytotoxic response that can result in a trade-off where tolerance of a pathogen may be associated with less organ injury.

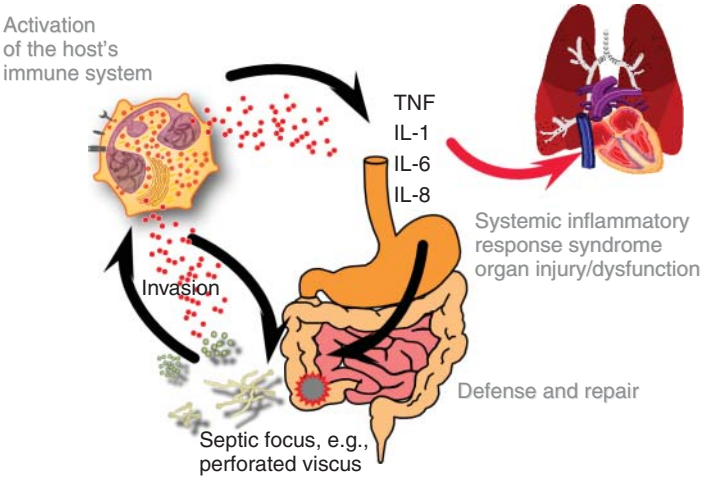

Figure 1.2 Activation of the innate immune system as a "double-edged sword." Activation of innate immunity reflects a prerequisite for defense and repair of a septic focus, such as a perforated viscus. However, this may lead to collateral damage if spillover of inflammatory mediators or release of activated cells into the systemic circulation occurs.

Table 1.1 Diagnostic criteria for the "systemic inflammatory response syndrome" (SIRS criteria).

- Temperature >38 or <36 °C
- Heart rate >90 beats/min
- Respiratory rate >20 beats/min or p_aCO_2 <32 Torr (4.3 kPa)
- White blood count >12 000 cells/mm^3 or <4000 cells/mm^3 or >10% immature (band) forms

molecular tools and the improved molecular and cellular understanding of the pathogen–host interaction via specific receptors and signaling cascades [5, 6]. As stated by Nathan [2]: "it makes no sense to use twenty-first century technology to develop drugs targeted at specific infections whose diagnosis is delayed by nineteenth-century methods." Thus, development of innovative diagnostic tests and strategies are needed to optimize treatment strategies, not only in selecting the correct anti-infective agent but also modulating inflammatory and other responses to fundamentally improve outcomes in a "personalized" manner.

The resulting diagnostic uncertainty regarding the causative pathogen reflects a central dilemma of intensive care physicians in treating life-threatening infections. On one hand, there is an important need to avoid delays in the initiation of appropriate antibiotics [7], yet this, in turn, triggers the overuse of "broad spectrum" antimicrobial agents creating a tremendous problem with multiresistant pathogens [8]. Likewise, many septic patients may already be in a state of overall immune suppression at the point of admission to intensive care, as anti-inflammatory systems are also activated in sepsis and these may outweigh the proinflammatory response. Introduction of an anti-inflammatory agent to such patients may arguably compromise the host even more.

1.2 Sepsis as a "Hidden Healthcare Disaster"

Sepsis arises from community-acquired infections but also, and more frequently, from healthcare-associated infections. It is a leading cause of morbidity and mortality worldwide. Its incidence is increasing, and the overall mortality is now in a similar range to that of myocardial infarction or stroke [9, 10]. This likely reflects changing demographics, with an aging population. In parallel, an ever-increasing number of invasive procedures, including those directly affecting the immune system, such as antineoplastic chemotherapy or organ transplantation, are performed in patients who would previously not have been considered for such procedures. As a consequence, the rate of hospitalization for sepsis in the United States increased from 221 per 100 000 population in 2001 to 377 per 100 000 in 2008 (Figure 1.3) [9]. A similar increase in the incidence of severe postoperative sepsis is also noted [11].

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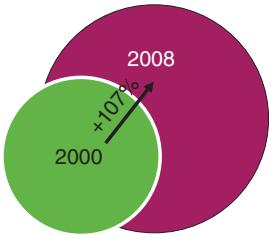

Figure 1.3 Sepsis – an underestimated and silently growing problem of modern healthcare: Because of multiple factors related to demographic changes, an increasing invasiveness of procedures in patients with inherent impaired immune function, and the advent of multidrug resistance in particular to Gram-negative pathogens, there is a silent but dramatic increase in the incidence of sepsis in healthcare systems across the world.

Sepsis has been called a "hidden public health disaster" [12]. Survivors carry an under-recognized risk of long-term cognitive and physical disability [13] and a more than twofold risk of dying over the next 5 years compared with appropriate controls [14]. The Center of Disease Control recently estimated that 15 billion dollars were spent on hospitalizations for sepsis alone in the United States and that inflation-adjusted aggregate costs for treating such patients increased annually by more than 10% [9].

1.3 Microorganisms and Types of Infection Triggering Sepsis

A recent global picture of infection and sepsis in ICUs worldwide is provided by the "Extended Prevalence of Infection in Intensive Care" (EPIC II) study. This reflects a 1-day, prospective, point prevalence study conducted on May 8, 2007, with subsequent follow-up [15]. Demographic, physiologic, bacteriologic, therapeutic, and outcome data were collected from approximately 14 000 patients in 1265 participating ICUs from 75 countries. These included 667 Western European ICUs, 210 Central and South American, 137 Asian, 97 Eastern European, 83 North American, 54 Oceanic, and 17 African. Sixty percent of participating ICUs were situated in university hospitals, 66% were mixed medical-surgical ICUs, and 94% had 24 h ICU physician coverage. On the study day, approximately half the patients were considered infected and 71% were receiving antibiotics. Infection was mostly of respiratory origin (66%), followed by abdomen (20%), bloodstream (15%), and renal tract/genitourinary system (14%). Microbiological cultures were positive in 70% of the patients with presumed infection, with 62% of positive isolates being Gram-negative organisms, 47% Gram-positive, and 19% fungi. The most common Gram-positive organism isolated was *Staphylococcus aureus* (20%), while the commonest Gram-negative organisms were *Pseudomonas* species (20%) and *Escherichia coli* (16%). Patients who had been in ICU for longer prior to the study day had higher rates of infection, especially with resistant and thus more difficult-to-treat pathogens, such as *Staphylococci*, *Acinetobacter*, *Pseudomonas*, and *Candida* species. Of note, ICU mortality (25% vs 11%) and hospital mortality (30% vs 15%) of infected patients was more than twice that of noninfected patients. Other, albeit smaller, surveys corroborate the EPIC II data and confirm the disease burden of infection in the critical care setting which increases with

the duration of stay as well as with shifting patterns of microorganisms [16]. Since 2007, a substantial increase in difficult-to-treat infections has been observed. This is primarily attributable to multidrug-resistant Gram-negative bacteria, a proportion of which are virtually untreatable, such as some carbapenemase-producing *Klebsiella* strains [17].

1.4 Emerging Problems Related to Resistance in Bacterial Infections

Methicillin-resistant *Staphylococcus aureus* (MRSA) remains an important cause of healthcare-associated infections, and is endemic in most hospitals. Healthcare-associated MRSA infections are associated with increased morbidity and mortality compared to infections caused by methicillin-susceptible strains [18, 19]. MRSA is also an increasingly important cause of infection in the community setting. MRSA infections, both healthcare- and community-associated, are generally caused by a very limited number of (clonal) strains, suggesting that most cases result from direct or indirect person-to-person transmission of MRSA [20, 21]. The major reservoir for transmission is likely to be infected or colonized patients, with the vector being healthcare personnel or contaminated, shared equipment. With the introduction of a variety of bundled strategies including, but not restricted to, careful hand hygiene, there has been an associated reduction in the burden of MRSA infection in the healthcare setting. In 2005, there were an estimated 94 000 MRSA infections in the United States associated with nearly 18 000 deaths. Of these, 86% were associated with healthcare delivery, two-thirds of which had their onset outside the hospital setting [22].

Despite measures that have successfully prevented and controlled healthcare-associated MRSA, the number of Gram-negative bacterial infections continues to grow [23]. This is compounded by an increasing problem of antibiotic resistance of these pathogens which may result in higher mortality and morbidity [24].

There are thus conflicting recommendations. On one hand, there is advice to administer appropriate and early empirical antibiotic therapy, especially in patients with risk factors such as compromised immune function. In view of the growing resistance problem, multiple broad-spectrum antibiotics are often proposed. On the other hand, there are strong recommendations to limit antibiotic usage in general. This is a clear testimony for the need of diagnostic tests with fundamentally improved performance regarding sensitivity, specificity, and, most importantly, time-to-result.

1.5 The Role of Fungi and Viruses

Bacteria dominate as the type of pathogen responsible for most life-threatening infections in the "immune competent" host. However, immunosuppression

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occurring in the later phases of sepsis [25], along with shifts in the host's commensal flora (primarily induced by antibiotics), contributes to overgrowth by fungi, most notably *Candida* species, in sterile body compartments. This can give rise to difficult-to-diagnose infections which may also contribute to the overall death toll. In a recent retrospective chart review study, we identified 999 patients with severe sepsis or septic shock from a total of 16 041 patients admitted to our 50-bed surgical ICU in a single center; hospital mortality was approximately 30% [16]. In total, data from 2117 blood cultures were available for analysis. Three phases could be described based on peaks in mortality. A third of all deaths occurred in the first 5 days following ICU admission. Of 882 blood cultures drawn within the first 5 days, only 15% were positive. Of note, 524 blood cultures were drawn in those patients staying >2 weeks and, while positive blood cultures were less frequently observed, the rate of opportunistic bacteria and *Candida* species doubled from 9% in the acute phase to 18% in this later phase.

While the role of invasive fungal infection is increasingly acknowledged, the contribution of viral infections to initiate or maintain a systemic inflammatory response syndrome SIRS is poorly defined. The 2009 influenza A (H1N1) pandemic did not significantly affect ICU occupancy rates and, compared with community-acquired pneumonia of other origins, H1N1 pneumonia was associated with the same risk of death when potential confounders were taken into consideration [26]. However, this pandemic more commonly affected young people, many of whom developed severe respiratory failure requiring extra-corporeal lung assist support. As the pathogen underlying ICU admission for community-acquired pneumonia is rarely identified with conventional diagnostics, viral infections are probably underdiagnosed. Viruses may play an important role in complicating the course of defined ICU patient populations, such as cytomegalovirus (CMV) in immunocompromised patients. While reactivation of dormant virus within the critically ill host likely occurs, it remains unclear to what extent they cause secondary infections. Antiviral treatment may improve outcome [27], but they do carry their own toxicity. Multicenter trials that address this problem are ongoing. Ganciclovir is frequently used as both first-line prophylaxis and systemic disease therapy against CMV, but resistance is increasingly occurring and this is associated with worse outcomes. Thus, implementation of rapid and sensitive techniques for the early detection and monitoring of CMV and ganciclovir resistance is clearly desirable to support patient management [28]. Furthermore, strategies to individualize the therapy of life-threatening infection by viruses such as CMV and Epstein–Barr virus may include, in addition to antiviral agents, either a reduction in immunosuppressant therapy (as these infections occur frequently in post-transplant patients [29]), or even immunoactivating agents such as Granulocyte macrophage colony-stimulating factor GM-CSF and interferon-gamma. Here, a reliable point-of-care monitoring of the host's immune system would potentially allow a correct selection of agents to improve clearance of infection while at the same time reducing the risk of, for example, graft failure in transplant patients [30].

1.6 The Need for New Approaches in Diagnostics of Life-Threatening Infection and Sepsis

The presence of an infectious focus is currently identified and confirmed by a combination of clinical examination and imaging techniques. Whenever possible, specimens are obtained from the site of infection in addition to blood cultures for conventional microbiology. An example is taking specimens during lung washings during bronchoscopy for suspected lower respiratory tract infection. At present, these techniques, though time consuming, allow a better determination of the infecting pathogen and antibiotic resistance patterns [31].

The polymerase-chain reaction (PCR) technique can directly amplify pathogen DNA from a suspected focus [32, 33] or a blood sample. This carries the potential to increase the sensitivity of pathogen detection and to decrease the result turnaround time in routine clinical practice [34]. At present, PCR testing is generally considered as supplementary to culture-based techniques, particularly for fastidious, resistant, and difficult-to-culture pathogens. Some experts suggest that the focus for such tests should be on detection of pathogens or resistance factors that fall outside guideline-recommended antibiotic coverage, as well as for specific at-risk populations, for example, transplant recipients [35]. However, with improvements in technology, point-of-care PCR testing of blood and other body samples may not only shorten the time to diagnosis but also reduce the number of patients receiving inappropriate empirical antibiotics.

Although inappropriate anti-infective therapy is seemingly associated with excess mortality [36, 37], a liberal or "aggressive" strategy with early initiation of (combined) antibiotics to cover a very broad spectrum of pathogens may also be associated with similar increases in mortality [38]. Although not fully understood, there is increasing support for the concept that concomitant release of host intracellular "danger-associated molecular patterns (DAMPs)" or "alarmins" [39] can signal, via the Toll-like and other receptor systems, a similar pathophysiological cascade culminating in multiple organ failure as that induced by "pathogen-associated molecular patterns (PAMPs)" released from bacteria and other pathogens [40, 41]. Indeed, DAMPs may be released even in the absence of pathogens or their PAMPs. The use of anti-infective agents themselves carries multiple adverse effects (Table 1.2); this is further compounded by altered handling of these drugs by the dysfunctional liver and/or kidney, which not only

Table 1.2 Problems associated with antibiotic use in individual patients.

- Overgrowth of (multi)-drug resistant bacteria and fungi
- Jarisch–Herxheimer reaction: release of bacterial products, such as endotoxin potentially triggering a vigorous host response with associated side effects on organ function
- Effects on critical cellular effector functions: immunomodulatory effects, impairment of mitochondrial function
- Typical drug-related side effects: for example, rashes, liver, and renal dysfunction

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makes pharmacokinetics and dosing unpredictable but also increases the risk of direct drug-induced toxicity [42].

1.7 Rapid and Sensitive Culture-Independent Strategies to Identify Blood Stream Infection

The basic principles regarding the diagnostics of infection hold particularly true for blood cultures, which is the current gold standard for identifying primary or secondary bloodstream infection. However, this is far from being an ideal gold standard, as a positive result is obtained in only a subset of severely septic patients and results are frequently obtained too late to influence clinical decision making [43, 44].

Molecular approaches to improve conventional culture-based identification may range from strategies to shorten the time from positivity of the blood culture to identification of the pathogen to complete culture-independent, direct microbial nucleic acid amplification techniques.

Important developments to improve the performance of the blood culture approach include fully automated instruments for handling and culture and the use of matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, which can decrease the time-to-result to approximately 1 h after a positive culture is recognized [44].

An alternative strategy, that is, extraction and amplification of microbial nucleic acids from a positive blood culture and subsequent hybridization on a microarray platform to detect the pathogen and certain resistance genes (gyrB, parE, and mecA) among 50 bacterial species (Prove-it Sepsis, Mobidiag, Helsinki, Finland), was recently evaluated [45]. A total of 2107 positive blood culture samples taken from 3318 blood samples from patients with suspected sepsis were analyzed; 86% of positive blood culture samples included a pathogen covered by the molecular assay which had an overall 94.7% sensitivity and 98.8%, specificity; both increased to 100% for identifying MRSA bacteremia. On average, the assay was 18 h faster than conventional blood cultures, providing proof of the concept that molecular assays can shorten the time-to-result. Shortcomings included an incomplete coverage of pathogens, an inability of the test to be applied directly to a biological sample, and restricted information regarding antimicrobial susceptibility (primarily regarding multiresistant Gram-negative bacteria) despite an excellent performance for detecting MRSA. While PCR-based detection of MRSA (and also Vancomycin-resistant Enterococci VRE) is feasible because of a limited number of resistance genes, the need to identify the large and continuously evolving set of genotypes encoding extended-spectrum β-lactamases renders a molecular approach difficult and a conventional PCR-based approach unreliable.

These shortcomings, obviously with the exception of the need for prior culture, also hold true for PCR-based approaches to directly amplify microbial nucleic acids from the bloodstream. This led to the view discussed earlier that PCR tests should only supplement but not replace blood culture (BC). However, such a

combined diagnostic approach has been documented in various clinical studies to yield higher detection rates of an alleged pathogen compared to each method alone and to decrease the time-to-result [34]. This appears to particularly hold true for well-defined at-risk populations and for fungal infection [46, 47].

PCR improves the identification of bloodstream infection because of typically easy-to-culture bacteria such as *Streptococci* and *Staphylococci*, but also in polymicrobial infection including difficult-to-culture species such as fungi [48–53]. As a result, and despite its inherent limitations, PCR may offer a promising approach to decrease the time-to-result, which may favorably influence antibiotic decision making in the critically ill septic host. Data from patients with sepsis, endocarditis, or bloodstream infection indicated an up to 2.5-fold increase in positive test results compared to blood culture. Concordance was moderate to good with respect to recovery of blood culture positive cases by PCR in most but not all studies applying the best validated technique, the LightCycler/Septifast® [1, 33]. In specific conditions, for example, infective endocarditis, concordance of results may be higher as the focus is within the bloodstream. It is noteworthy that a significant proportion (approximately 20–30%) of blood culture findings was not reproduced by PCR [54]. In addition to the limited use of PCR to comprehensively assess resistance patterns and the inherent high costs of broad, multiplexed PCR tests, these findings prompted the recommendation that PCR be viewed at present as a supplementary test rather than as a replacement for conventional blood culture testing [35].

Several avenues have been pursued to address the opposite problem, that is, a PCR positive but blood culture negative result. One study analyzing microbiological specimens other than blood confirmed that PCR performed on DNA prepared from whole blood did increase the number of presumably true-positive results in septic patients [55]. However, a proportion of positive PCR results still remained unconfirmed by other tests. In this study, 200 adults with presumed infection presenting to an emergency room had 45 positive PCR tests compared to 37 positive blood cultures. Sixty-eight percent of the positive PCR results were confirmed by blood, urine, or catheter cultures. Consistent with earlier discussed results, all MRSA bacteremia episodes were detected.

Assessing the association of PCR status with measures of morbidity and mortality reflects a complementary strategy to validate the significance of an amplicon in the absence of a concomitant blood culture result. We studied cases with severe sepsis, and found 34.7% of PCRs tests were positive compared to 16.5% of blood cultures [54]. Seventy-eight percent of positive cultures had a corresponding PCR result, while only 23% of PCR results were confirmed by blood culture. Compared to patients with a negative PCR, those testing positive had higher organ dysfunction scores and a trend toward higher mortality (39.1% vs 25.3%; $p = 0.115$). This held true particularly for predefined cohorts of patients with a fungal amplicon. These were observed twice as frequently as cultures that were positive for fungi; both results were associated with an extremely high mortality (90%). These data lend support to the concept that the presence of a pathogen-associated DNA amplicon reflects a meaningful event in severe sepsis. As a consequence, PCR

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warrants further investigation as to its suitability to guide anti-infective therapy in the light of the conflicting findings of initiation of early "aggressive" antibiotic therapy [37, 38]. Such studies are currently under way. Thus, PCR may serve to close the gap regarding those infections not covered by guideline-recommended initial treatment [35], but most likely will not allow a fundamental improvement in diagnostics such that antibiotic therapy can be safely narrowed in terms of spectrum of activity or even withheld.

Although recovery rates of PCR are generally higher than those obtained from conventional culture, in most cases a causative microorganism cannot be identified, even in the most severely sick septic patients. This has prompted efforts to improve pre-analytical handling of blood samples to increase sensitivity. Whole blood as a template for PCR faces limitations because of its very high human DNA background. Increasing the ratio of pathogen to host DNA may improve the diagnostic performance by amplifying pathogen DNA against the eukaryotic background. Discrimination of "self" as opposed to bacterial DNA is achieved by immunocompetent cells via species-specific cytidylate-phosphate-deoxyguanylate (CpG) motif recognition via TLR9 (toll-like receptor). This process is not restricted to TLR9 but is also observed, for example, for a human cytidylate-phosphate-deoxyguanylate-binding protein (CXXC Finger Protein 1 or hCGBP) [56, 57]. This mammalian transcriptional activator avidly binds unmethylated CpG dinucleotid motifs by recognition of the sequence [A/C]CG[A/C] [57] with an even higher number of potential binding sites compared to TLR9 [58, 59]. A cloned, truncated protein derived from CXXC Finger Protein 1 can be used for affinity chromatography to decrease the human DNA background in blood samples taken from septic patients. When comparing these results to blood culture, approximately a threefold increase in sensitivity was achieved in a limited cohort of patients [60]. Time-to-result for episodes of bacteremia or "DNAemia" was also reduced, as in other PCR-based approaches, to <8 h despite the additional preanalytic step. However, the full potential of this test, which requires substantial "hands-on-time," can only be exploited if staffing of the laboratory allows unscheduled access, or automated systems become available [34].

1.8 Beyond Infection – Profiling the Immune Response of the Septic Host

In addition to identifying the alleged pathogen, biomarkers reflecting the response of the innate immune system are required to consolidate a diagnosis of "sepsis." These biomarkers may range from individual biomolecules, for example, proteins such as procalcitonin (PCT), to multiplexed signatures of biomolecules including transcriptomic, proteomic, or metabolomic profiles [61]. Profiling of the host response can provide valuable information regarding the pathogen, prognosis, and potential response to treatment [62]. PCT is the best validated single protein marker to date; while this marker can be used to identify a population at risk and

to guide anti-infective therapy [63], it is far from perfect, so there is clear space for superior technologies.

As mentioned above, microorganisms bear well-conserved structures known as PAMPs. These bind to the pattern recognition receptors (PRRs) located either on the cell membrane or in the cytosol of host cells to initiate the host response. Well-recognized PAMPs include lipopolysaccharides (LPSs) found in the outer membrane of Gram-negative bacteria, peptidoglycan, and muramyl dipeptide (MDP), which are cell wall constituents of Gram-positive bacteria, as well as bacterial flagellin and nonmethylated CpG DNA motifs. The various families of PRRs currently comprise TLRs, NOD-like (nucleotide-binding oligomerization domain-like) receptors (NLRs), and RIG-like (retinoic acid-inducible gene-like) receptors (RLRs). While not totally specific, TLR2 recognizes peptidoglycan monomers, TLR4 recognizes bacterial LPS, TLR5 recognizes bacterial flagellin, TLR9 recognizes CpG DNA motifs, and NLRs recognize MDP [41]. The current pathogenetic view of sepsis is based mainly on the modulation of proinflammatory and anti-inflammatory cytokine production by the interaction of PAMPs with these PRRs, which, in turn, is modulated by the host genomic background.

Emerging data lend support to the notion that compound markers utilizing parallel "omics" approaches may describe the host response in a more comprehensive way to individualize treatment [64–66]. Recent data from transcriptomic, proteomic, and metabolomic profiling indicate that marker panels are superior to single genes or markers, and can differentiate noninfectious from sepsis-associated systemic inflammation [67, 68]. This is achieved by reflect signaling via the various PRRs that are characterized by shared or "common host response" patterns as well as by specific responses to the pathogen. Thus novel and robust "biomarkers" are feasible and are urgently needed to identify patterns associated with infection as the underlying cause of a systemic host response in a correct and timely manner. Proof-of-concept studies show that algorithms can be developed to describe gene-expression signals, for example, as continuous, nondimensional scores, or as trajectories to assess infectious or noninfectious causes for organ dysfunction.

1.9 Host Factors Contributing to Pathogenesis of Sepsis

The prognosis of the patient depends on both the virulence of the pathogen and the individual host's response to infection that is directed to kill the invading pathogens, but is also affected by host (genetic) factors [69]. None of the numerous interventions developed to modulate the host response, such as cytokine-neutralizing therapies, that were documented to improve survival in preclinical models of sepsis have proved successful in subsequent clinical studies [50]. Although inclusion of patients into these trials has not been stratified according to biomarkers, *post hoc* analysis suggests that a signal indicating a beneficial effect of neutralizing mediators of inflammation may be associated with markers of activation of the immune system, such as IL-6. Concepts referred

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to as *theragnostic strategies*, using a biomarker (set) to guide therapeutic decision making, are promising but in their infancy. Currently discussed or available interventions include a broad range of drugs that can interfere with innate or adaptive immunity to either inhibit an overwhelming inflammatory response or to enhance this vital defense mechanism. A current paradigm holds that a (late) phase of immune paralysis follows an initial "cytokine storm" [70, 71]. Acknowledging that reasons for failure are likely to be multifaceted, the inability to individually stratify patients by their host response is most probably a key factor. Indeed, the same nonspecific SIRS criteria, which essentially rely on a clinical physiological phenotype, have been used to include septic patients into trials of immunomodulatory strategies. This approach is increasingly viewed as fundamentally flawed. Novel and robust "biomarkers," most likely panels of biomolecules, are urgently needed to correctly and timely describe the nature of the host response and the immune status of an individual patient. Specifically, there is an imperative to monitor multiple biomarkers related to pro- as well as anti-inflammatory gene products. This is supported by the ever-increasing evidence base from transcriptomic studies showing that events are not tightly associated with "early" and "late" phases of the sepsis disease continuum. Activation of inflammatory innate immunity occurs alongside impaired adaptive immune responses [72].

A phenotypic characterization to enable individualization of therapy may also require a description of organ function beyond current strategies that simply measure markers of injury, such as enzymes released upon cellular demise, or metabolites, such as creatinine or bilirubin, that monitor organ dysfunction as a hallmark of severe sepsis. These indicators of injury and function are rather

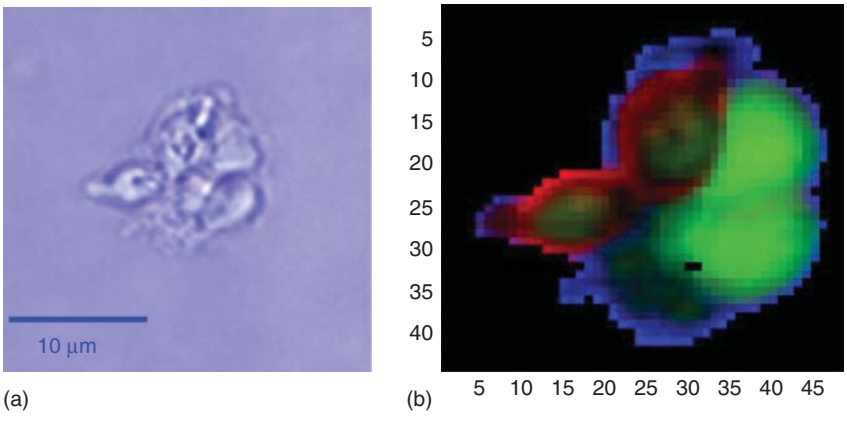

Figure 1.4 Biophotonic strategies to study infection and ensuing host response. Micro-Raman spectroscopic image of *Candida albicans* phagocytosed by a neutrophil. This allows the identification of the pathogen and its dimorphic behavior (hyphal as opposed to yeast phenotype) that impacts on its pathogenicity. Such information can be retrieved label-free on a single-cell level without the need for culture. (a) White-light image and (b) false-color Raman image constructed by end-member analysis using the N-finder algorithm (blue: neutrophil, red: phagocytosed Candida in hyphal form, and green: phagocytosed candida in yeast form).

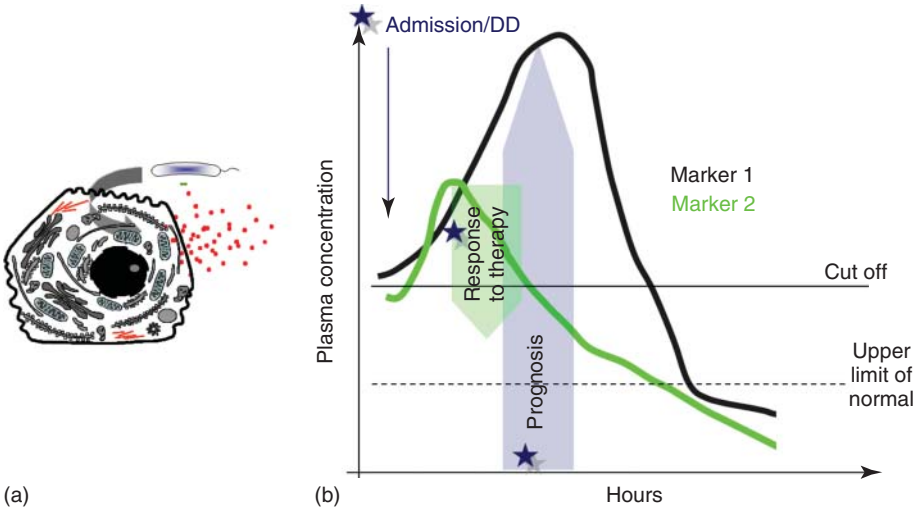

Figure 1.5 The use of "biomarkers" released upon contact of host cells with pathogens to diagnose sepsis and guide therapeutic interventions. (a) Biomarkers are released in response and, thus, indicate contact of pathogen(s) or pathogen-related compounds with host cells. (b) Kinetics of release of two virtual biomarkers (marker 1 and 2). Because of their differing release kinetics, these markers could be used to derive information regarding the nature of disease, prognosis, and response to treatment. These data could then be used to guide therapeutic interventions, such as initiation or discontinuation of antibiotics in case of infection.

insensitive compared to metabolomic parameters that are increasingly available with the advent of mass spectrometry in the routine laboratory.

Taken together, despite the moderate progress achieved with quality management programs such as the "Surviving Sepsis Campaign" [31, 73], sepsis remains an underappreciated killer. As current improvement strategies are directed, in the light of diagnostic uncertainty, at early administration of broad-spectrum antibiotics, development of (multi)-drug antimicrobial resistance has become a major concern. Moreover, the host response is subject to significant variability, with a vulnerable trade-off between disease tolerance and pathogen elimination. Unmet medical needs and strategies to improve patient care will most likely be achieved via improved diagnostics to identify the pathogen and to describe the immune status early and in a way that tailored use of available anti-infective and immunomodulatory therapeutics is made possible (Figures 1.4 and 1.5).

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2
Identification Methods – An Overview

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2.1
Taxonomy of Pathogenic Organisms

The morphologic classification was the very first method to differentiate the microorganisms based on microscopic observation. Around the year 1675, Antoni van Leeuwenhoek constructed the first microscopes, which were very simple instruments with soda lime glass lenses made by a melting process. He achieved already a 500-fold magnification and could differentiate the morphology of some microorganisms (such as cocci or bacilli (rods)). However, his work was hardly respected and received little attention at that time. Thus, he kept the secret of how he created the lenses, and for a long time no one was able to reconstruct his microscopes.

Modern bacteriology/microbiology began in the nineteenth century with the development of high-performance microscopes using achromatic optical lenses, and pioneers like Ferdinand Cohn, Louis Pasteur, and Robert Koch are considered the founders of these disciplines.

While the first taxonomy of microorganisms was related to morphologic properties, which are still anchored in the nomenclature (e.g., *Streptococcus* spp. or *Rhodospirillum* spp.), modern bacteriology discovered different metabolic processes that can be used to differentiate organisms and are more suitable to classify their taxonomy. Modern genetics, which began with the works of Gregor Johann Mendel in the mid-nineteenth century, opened new prospects, and in 1977 Carl Richard Woese published the most influential work of the phylogenetic taxonomy based on 16S RNA [1].

Presently, species differentiation of the (pathogenic) organisms is performed by their morphologic (microscopy) and metabolic properties (culture-based) as well as genetically (NAT (nucleic acid amplification technique)-based). Thereby, microscopy and culture-based differentiation is still quite similar to those procedures performed in nineteenth century by Koch and coevals!

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It is essential to understand the taxonomic relationship of the pathogenic organisms to choose the proper technique or test to differentiate the species. Therefore, a rudimentary but fundamental overview of the most related microbial pathogens and their classification into the taxonomic neighborhood are given in Appendix

2.2 Microscopic Methods

Although many different methods are now available to identify and differentiate bacteria, microscopic methods still occupy an important place in clinical practice. Culture-based methods have some limitations: (i) they cannot distinguish between colonization or tissue invasion, and many opportunistic bacteria, fungi, and viruses are natural commensals in human compartments; (ii) some of the invasive species are noncultivable by routine diagnostic methods; and (iii) others are slow-growing species, limiting the method. In addition to identifying the pathogen, microscopic methods provide information about tissue damage and the presence and type of inflammatory cells. On the other hand, tissue samples from surgical specimens are sometimes directly placed in fixatives, thereby making culture analysis impossible. Furthermore, in case of urgent diagnosis, for example, of meningitis, fast microscopic methods are still very important in clinical practice.

2.2.1 Stains

Since microorganisms yield only weak contrasts for direct light microscopic analysis, staining has to be used to improve the performance. Differential stains are common in routine diagnostics because they yield some information about the species and are faster compared to medium-based or automated species differentiation methods. Microscopic methods are, however, laboratory-intensive and error-prone and thus require well-trained personnel to avoid misinterpretations of the data, and a profound knowledge of infectious diseases and histopathology to apply the correct staining technique for the suspected pathogen. Here, only a selection of the most common staining methods is presented; for more details, see reviews on the subject [2, 3]. Some of the stains described in the following are suitable for bacteria, fungi, human cells, as well as parasites; others are more specific for a particular type of pathogen.

The *Gram-stain* is an old (first published in 1884 by Hans Christian Gram) but the most popular method to distinguish between Gram-positive and Gram-negative bacteria. In this method, the bacterial cell wall of Gram-positives becomes stained by crystal violet and iodine which form an insoluble blue dye complex. Gram-negatives are counterstained by fuchsin (pink) or safranin (red). This method is based on the different cell wall structures of both bacteria types. Gram-positives exhibit a thick peptidoglycan (murein) which dehydrates by

Appendix: Taxonomy

Bacteria

Phylum	*Class*	*Order*	*Family*	*Genus*	*Species*
Gram negative bacteria					
Proteobacteria	***Betaproteobacteria***	*Bulkholderiales*	*Bulkholderiaceae*	*Bulkholderia:*	***B. cepacia***
			Alcaligenaceae	*Bordella*	***B. pertussis***
				Achromobacter	***A. xyloxidans***
		Neisseriales	*Neisseriacaea*	*Neisseria*	***N. gonorrhoeae*** ***N. menengitidis***
	Gammaproteobacteria	*Pseudomonales*	*Pseudomonaceae*	*Pseudomonas*	***P. aeruginosa*** ***P. fluorescens***
			Moraxelaceae	*Acinetobacter*	***A. baumanii***
		Vibrionales	*Vibrionaceae*	*Vibrio*	***V. cholarae***
		Enterobacteriales	*Enterobacteriaceae*	*Escherichia*	***E. coli***
				Klebsiella	***K. pneumoniea*** ***K.oxycota***
				Proteus	***P. mirabilis***
				Salmonella	***S. enterica*** ***S. thyphi*** ***S. typhimurium***
				Yersinia	***Y. pestis***
		Pasteurellales	*Pasteurellceae*	*Heamophilus*	***H. influenzae***
		Legionaellales	*Legionellaceae*	*Legionella*	***L. pneumophila***
			Coxiellaceae	*Coxiella*	***C. burnetii***
				Rickettsiella	
	Epsilonproteobacteria	*Campylocacteriales*	*Helicobactreriaceae*	*Helicobacter*	***H. pylori***
Actinobacteria	***Actinomycetales***	*Actinomycetaceae*	*Mycobacteriaceae*	*Actinomyces*	
		Corynebacterineae	*Mycobacteriaceae*	*Mycobacterium*	***M. tuberculosis*** ***M. avium***
			Nocardiaceae	*Nocardia*	

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Appendix: Taxonomy

Gram-positive bacteria					
Furmicutes	***Bacilli***	*Bacilliales*	*Listeriaceae*	*Listeria*	***L. monocytogenesi***
			Staphylocaccaceae	*Staphylococcus*	***S. aureus***
					S. epidermidis
		Lactobacilliales	*Enterococcaceae*	*Enterococcus*	***E. feacium***
					E. feacalis
			Streptococcaceae	*Streptococcus*	***S. pneumoniae***
					S. pyrogenesi

Appendix: Taxonomy

Fungi

Phylum	*Class*	*Order*	*Family*	*Genus*	*Species*
Ascomycota	***Eurotiomycetes***	*Eurotiales*	*Trichocomaceae*	*Aspergillus*	*A. fumigatus* *A. fischerianus* *A. clavatus* *A. flavus*
				Penicillium	*P. marnefffei*
		Onygenales	*Onygenaceae*	*Coccidioides*	*C. immitis* *C. posadasii*
				Histoplasma	*H. capsulatum*
			Ajellomycetaceae	*Paracoccidioides*	*P. brasiliensis*
				Blastomyces	*B. dermatitidis*
	Sordariomycetes	*Hypocreales*	*Nectriaceae*	*Fusarium*	*F. solani* *F. oxysporum* *F. verticilliodes* *F. proliferatum*
		Ophiostomatales	*Ophiostomataceae*	*Sporothrix*	*S. schenckii*
	Saccharomycetes	*Sacchromycetales*	*Saccharomycetaceae*	*Saccharomyces*	*S. cerevisiae*
				Candida	*C. albicans* *C. glabrata*
	Pneumocystidomycetes	*Pneumocystidales*	*Pneumocystidaceae*	*Pneumocystis*	*P. jirovecii*
Basidomycota	***Tremellomycetes***	*Tremellales*	*Tremelacaea*	*Cryptococcus*	*C. laurentii* *C. albidus* *C. neoformans* *C. uniguttulatus*
			Trichiosporonaceae	*Trichiosporon*	*T. ovoides* *T. mucoides* *T. inikin* *T. cutaneum* *T. dohaense* *T. loubieri* *T. asahii* *T. asteroides* *T. dermatis*

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Appendix: Taxonomy

Parasites

Phylum	*Class*	*Order*	*Family*	*Genus*	*Species*
Platyhelminthes	***Trematoda (subclass Digenea)***	*Strigeidida*	*Schistosomatidae*	*Schistosoma*	
	Cestoda	*Cyclophilidea*	*Hymenolepididae*	*Hymenolepis*	***H. nana*** ***H. diminuta***
			Taeniidae	*Taenia*	***T. saginata*** ***T. solium***
				Echinococcus	
Nematoda	***Secernentea***	*Ascaridida*	*Ascarididae*	*Ascaris*	***A. lumbricoides***
		Spirurida	*Strongylidae*	*Strongyloides*	***S. stercoralis***
			Onchocercidae	*Wuchereria*	***W. brancrofti***
				Brugia	***B. mylayi*** ***B. timori***
				Loa	***L. loa***
				Mansonella	***M.streptocerce*** ***M. perstans*** ***M. ozzardi***
				Onchocerca	***O. volvulus***
		Rhabdita	*Trichostrongylidae*	*Trichostrongylus*	
		Oxyurida	*Oxyuridae*	*Enterobius*	
	Adenophorea	*Trichocephalida*	*Trichuridae*	*Trichuriss*	***T. trichiura***
			Trichinellidae	*Trichinella*	***T. spiralis***
Acanthocephala	***Archiacanthocephala***	*Moniliformida*	*Moniliformidae*	*Moniliformis*	***M. moniliformis***

Appendix: Taxonomy

Viruses

Group	Family	Subfamily or Genus	Species
dsDNA	*Herpesviridae*	*α-Herpesvirinae*	*Simplexvirus (HHV-1, HHV-2)* *Varicellavarus (VZV / HHV-3)*
		β-Herpesvirinae	*Cytomegalomvirus (HCMV / HHV5)* *HHV6, HHV-7*
		λ-Herpesvirinae	*Epstein-Barr Virus (EBV / HHV-4)* *Kaposi's sarcoma-associated herpesvirus (KSHV / HHV-8)*
	Adenoviridae	*Mastadenovirus*	*Human adenovirus A to G*
	Papillomaviridae	*Alphapapillomavirus Betapapillomavirus* *Gammapapillomavirus Mupapillomavirus* *Nupapillomavirus*	
	Poxviridae	*Orthopoxvirus* *Parapoxvirus* *Yatapoxvirus* *Molluscipoxvirus*	
ssDNA	*Anelloviridae*	*Alphatorquevirus*	*Torque teno virus (TTV)* *Torque teno midi virus (TTMDV)* *Torque teno mini virus (TTMV)*
dsRNA	*Reoviridae*	*Rotovirus* *Orbivirus*	
(+)ssRNA	*Coronaviridae*		
	Picoronaviridae	*Heptovirus*	*HAV-1*
		Rinovirus	
		Enterovirus	*Poliovirus*
	Astroviridae		
	Calciviridae	*Norovirus*	
	Flaviviridae	*Flavivirus*	*Dengue virus*
		Hepacivirus	*Hepatitis C virus*
	Hepeviridae	*Hepevirus*	*Hepatitis E virus*
(-)ssRNA	*Bornaviridae*		
	Filoviridae		
	Paramyxoviridae	*Paramyxovirinae*	*Marbllivirus (Measles)* *Rubulavirus (Mums)*
		Pneumovorinae	*Pneumovirus*
ssRNA-RT	*Retroviridae*	*Lentivirus*	*HIV*
		Deltaretrovirus	*HTLV*
dsDNA-RT	*Hepadnaviridae*		*Hepatitis B virus*

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Appendix: Diagnostic characteristics

Genus	Morphology			Growth		Diagnostic
	Gram	Shape	Capsulation	Respiration	Medium	
Bordetella	-	coccobacilli	+	aerobic	Regan-Lowe agar	*B. pertussis*: direct immunofluorescence, PCR amplificatioy
Borrelia	-	spirochete		anaerobic	rarely cultured	*B. burgdorferi*: microscopy (Giemsa or Wright), PCR, serology
Brucella	-	coccobacilli	-	aerobic	blood agar	agglutination serology
Campylobacter	-	spiralbacillus	-	facultative	blood agar	Gram stain and microscopy
Chlamydia/Chlamydophila	-	small, round rods	-	obligate anaerobic		*C. trachomatis*: immunofluorescense, ELISA, DNA hybridization *C. psittaci*: indirect immunofluorescence, complement fixation
Clostridium	+	large rods	+	facultativ anaerobic	anaerobic blood agar	*C. difficile*: ELISA *C. perfringens*: double-zone ß-hemolysis
Corynebacterium	+	slender rods	-	facultative anaerobic	Tinsdale agar	*C. diphtheriae*: immunologic precipitin reaction
Enterococcus	+	slender rods		facultative anaerobic	bile-esculin agar	Hydrolyze esculin in presence of bile
Escherichia	-	short rods	-/+	aerobic anaerobic	Mac Conkey	Ferment products and metabolic activity.
Francisella	-	cocco-bacillus	+	strictly aerobic	rarely cultured	*F. tularensis*: serology
Haemophilus	-	cocco-bacillus	-/+		choclate agar	*H. influenzae*: quellung reaction, immonofluorescence, detection of capsular antigen
Helicobacter	-	curved rods		microaerophile	medium containing antibiotics against fecal flora	*H. pylori*: urease-positvity by radioactively labeled urea
Legionella	-	slender rods	-	aerobic	specialized medium	*L. pneumophila*: direct immunofluorescence, radioimmunoassay, hybridization to ribosomal RNA using DNA probe
Leptospira	-	long rods		strictly aerobic	specialized medium	*L. interrogans*: dark-field microscopy, agglutionation tests
Listeria	+	short rods		facultative anaerobic	Columbia blood agar	*L. monocytogenes*: β-hemolysis and Catalase production on blood agar

Appendix: Diagnostic characteristics

Mycobacterium		long, slender rods	-	aerobic	Lowenstein-Jensen agar	*M. leprae*: acid-fast staining *M. tuberculosis*: Ziehl-Neelsen, hybridization probes
Mycoplasma		singly or in pairs	+	facultative anaerobic	rarely cultured	*M. pneumoniae*: complement fixation test, DNA probes
Neisseria	-	diplococci	-/+	aerobic	Thayer-Martin agar	*N. gonorrhoeae*: oxidase test, fermentation of glucose *N. meningitidis*: chocolate agar, oxidase test
Pseudomonas	-	rods	+	obligate aerobic	Mac Conkey	Production of pyocyanine and fluorescein, oxidase test
Rickettsia	-	pleomorphic		aerobic	non-culturedin media (but in chicken embryos)	*R. rickettsii*: immunofluorescence, serlogy
Salmonella	-	bacilli	+	facultative anaerobic	Mac Conkey	colorless on Mac Conkey *S. typhi*: serology against O-antigen
Shigella	-	rods	-	facultative anaerobic	Hektoen agar	*bluish-green on HE agar* *S. sonnei*: ferments lactose, indole negative *S. flexneri*: *gas positive*
Staphylococcus	+	round cocci	-/+	facultative anaerobic	enriched medium	*S. aureus*, *S. epidermidis*, *S. saprophyticus*: catalase test, coagulase test, novobiocin
Streptococcus	+	ovoid to spherical, pairs or chains	-/+	facultative anaerobic	blood agar	*S. agalactiae*: catalase test, hydrolyes sodium hippurate *S. pneumoniae*: quellung reaction, optochin *S. pyogenes*: β-hemolysis, serology, bacitracin
Treponema	-	long rods	-	aerobic	none	*T. pallidum*: dark field microscopy, serology including non/treponemal test
Vibrio	-	short rods	-	facultative anaerobic	Mac Conkey	Ferment products and metabolic activity, agglutination test, serology
Yersinia	-	small rods	+	facultative anaerobic	Mac Conkey	Ferment products and metabolic activity, ELISA, serology

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decolorizing with ethanol, thereby enclosing the dye complex. Gram-negatives have only few murein layers but are enclosed by an outer membrane made of lipopolysaccharides (LPSs) and lipopeptides which lose their integrity by adding ethanol, releasing the dye complex. Since human cells are also stained, this method might provide information about tissue damage and immune response. But the Gram-stain may also stain some fungi, such as *Candida* spp., *Nocardia* spp., or *Actinomyces* spp. False Gram-negatives might occur when the Gram-positive cells are old or dead, or the cell wall is damaged, or if the destaining process was too long and strong. Thus specificity of this method is limited.

Methylene blue is suitable to visualize the cell forms of the bacteria and their location in tissues, but is not appropriate for their differentiation. Methylene blue dye stains primary proteins and therefore is not specific for microorganisms.

To visualize capsular cell walls, *India ink* can be used. Thereby, the background appears dark whereas the ink pigments do not enter the capsular compounds, resulting in light spheres surrounding the cells.

Bacteria that contain unusual lipids, such as mycolic acids (*Mycobacterium* and *Nocardia* are the so-called *acid-fast* species), and can be stained by carbol-fuchsin (according to *Ziehl–Neelsen stain* or *Kinyoun stain*), a phenolic, lipophilic compound. The decolorization of nonacid-fast cells is performed by acid alcohol. For counterstaining, methylene blue or malachite green can be used. This is a suitable method for the identification of *Mycobacteria* that cause tuberculosis in sputum or tissues.

Hematoxylin and eosin (H&E) stain is also suitable for visualizing some microorganisms, but it is primarily used as a counterstain to evaluate the histopathology of tissues. Since it stains the nuclei and cytoplasm of eukaryotic cells, fungi can easily be overlooked by this method. Thus other fungal stains are recommended, such as *Grocott-(Gomori) methenamine silver* (*GMS*) and *periodic acid-Schiff* (*PAS*) stains. Both visualize various fungi by oxidation of the mucopolysaccharides of fungal cell walls, thereby reducing silver nitrate to metallic silver which appears dark or black in GMS, or the Schiff-reagent (fuchsin dye) which appears as purple-magenta in PAS. The tissue can be counterstained by H&E (stains cell nuclei blue, and cytoplasm red or pink) or basic stains such as light green (stains collagen). GMS also stains *Nocardia*, *Mycobacteria*, and some other bacteria with polysaccharide capsules (such as *Streptococcus pneumoniae*) but also amoeba. However, the Gridley's method is more suitable to visualize amoeba. The GMS stain masks the pigments of some fungi which could be relevant for diagnosis.

Calcofluor white is a blue-white fluorescent stain that binds to cellulose and chitin in the fungal cell walls. It can be used in combination with KOH or with *Papanicolaou stain* (a multichromatic stain with compartment-specific dyes) to increase the efficiency. The *KOH test* is one of the easiest fungal tests and can be performed on a variety of materials such as hair, skin, or nail samples or scrapings. The specimen is placed on a microscope slide and covered by 10 or 20% KOH to eliminate keratin and protein-rich human cell debris. The fungal cells can be

directly observed under a microscope or be additionally stained with lactophenol cotton blue or calcofluor.

The *Giemsa stain* was primarily developed to visualize malaria parasites (*Plasmodium* spp.) at the end of the nineteenth century. The Giemsa's solution is a composition of methylene blue and different oxidation products of methylene blue (Azure A and B) [4]. This is a multiple staining technique that visualizes different compartments and cell structures in different colors using one stain. It is used to visualize parasites, fungi, and some bacteria, such as *Helicobacter pylori*, and also different types of granulocytes and other blood cells revealing also their basophilic and eosinophilic properties. The main disadvantage is that the stain vanishes fast.

Viruses cannot be determined by staining itself, but they can lead to characteristic changes in the host cells and tissues, such as the formation of inclusion bodies in the nucleus and/or cytoplasm, formation of multinucleate giant cells, presence of a perinuclear halo, lymphocytic infiltration, or necrosis. These changes can be visualized by appropriate tissue stains such as the Giemsa stain. The most commonly recognized viral infections that can be recognized by histopathologic changes are those caused by herpes viruses such as HSV (Herpes simplex virus) and CMV (Cytomegalovirus). However, immunohistochemistry (IHC) is more specific and sensitive and therefore histopathologic methods for virus diagnostics are uncommon.

Parasites, mainly the ova and cyst, can be easily be recognized by a saline mount or an iodine-stained wet mount. Usually a conformation is performed by the Giemsa stain, which is suitable for many parasites such as *Plasmodium* spp., *Trypanosoma* spp., *Leishmania* spp., or *Toxoplasma gondii* (and others). Also H&E or PAS stains are suitable for detecting parasites and to visualize tissue and cell morphology.

2.2.2 Autofluorescence

Organisms contain many optically active molecules that can absorb light in the UV–visible region of the spectrum, and some of them emit light of higher wavelengths that can be detected as autofluorescence. This endogenous autofluorescence has to be distinguished from the fluorescent signals obtained by adding exogenous markers. The main fluorophores in cells are aromatic amino acids, lipo-pigments and pyridinic coenzymes (NADP(H)), and flavins. Autofluorescence offers many benefits. It can be performed in real time without fixing or staining of the specimens and yields additional information about the morphological and physiological state of cells and tissues. However, autofluorescence is still not commonly used as a routine diagnostic in clinical microbiology. Here, it often impedes other microscopic techniques, but the effect can be reduced by treatment, for example, with sodium borohydride.

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2.2.3 Immunofluorescence

Immunofluorescence (IFT) and IHC refer to the antibody – antigen recognition for the detection of cellular molecular markers (mainly proteins or polysaccharides) and thus they combine the sensitivity and specificity of immunology with the precision of microscopy. Different conjugates, such as peroxidase (POD), APase, or fluorophores, that are bound to the antibody offer a wide range of microscopic methods and detection options, such as luminescence, fluorescence, or colorimetric measurements.

IFT can be easily applied routinely to formalin-fixed, paraffin-embedded tissues sections, and allows differentiation of morphologically similar cells or microorganisms. This technique is increasingly used for the diagnosis and classification of carcinomas (IHC) and is common in the detection of bacteria (IFT). In some cases, such as endocarditis or gastric infections, it improves the diagnostic accuracy of fastidious species such as *Tropheryma whipplei* [5], *Coxiella burnetii* [6], or *Helicobacter pylori* [7]. On the other hand, it is a suitable tool for the determination of some viruses (e.g., HSV, CMV, EBV, adenoviruses, or parvovirus B19) because they offer specific antigens that can be used as AK targets [8]. Also some fungi, such as *Candida*, *Aspergillus*, *Blastomyces*, *Cryptococcus*, *Histoplasma*, *Coccidioides*, *or Pneumocystis*, and a few parasites, such as *Leishmania*, can be identified by IFT.

IFT can be performed by monoclonal and polyclonal antibodies, using a direct (one antibody) or indirect (two antibodies, the first specific for the antigen, and the second labeled and specific for the first antibody) protocol. Some AKs are commercially available, but they can cross-react with other organisms, thereby strongly affecting the results, which is their main disadvantage. This method is also limited by the availability of specific antibodies and the preservation of appropriate antigen epitopes.

2.2.4 *In Situ* Hybridization

In situ hybridization (ISH) is based on specific nucleic acid sequences (mostly RNA) within the sample which hybridize with complementary DNA probes. When fluorophores are coupled to the probe, it is usually called *FISH* (Fluorescence *in situ* hybridization) (detailed description of the FISH technique is provided in Chapter 7). The genetic code contains unique sequences that are specific for a genus, species, or even subspecies or strain; thus it provides a powerful tool also for epidemiologic studies. Moreover, it has been reported that it is sufficient to detect single genes with a low copy mRNA per cell [9]. This method is applicable for all types of cells: host, bacteria, fungi, viruses, and parasites. Targets can be chosen and labeled individually because of the commercially available synthesis and modification, but it requires the knowledge of the particular sequence and further validation of the specificity. Presently,

customer services offering individual design of probes and assays are on the rise.

ISH represents a complementary technique that bridges the gap between morphologic, immunologic, and molecular techniques. ISH shares some advantages with the IHC, such as high sensitivity and specificity with low time effort and relatively low cost. Both methods are also applicable on formalin-fixed and embedded samples, but with somewhat reduced sensitivity, thereby enabling retrospective analysis for several decades. For diagnostic approaches, often 16sRNA is targeted, because its high copy number increases the sensitivity. However, this method is presently not routinely used in clinical diagnostics.

2.2.5 Dark-Field Microscopy

Dark-field microscopy uses crossed light beams to illuminate the sample. Such light beams can be obtained by a central aperture which generates a ring of light. The unscattered beam is excluded from the image of the diffracted light. Thus, the objects of the sample appear bright on a dark background. This method is simple but shows higher quality and increased contrast compared to direct light microscopy and is therefore suitable for live and unstained biological samples. However, interpretations should be done with care because some features might be invisible in the dark field image.

This method is often used for the detection of the fragile bacteria *Treponema pallidum*, the causative of syphilis.

2.3 Culture-Based Methods

Culture-based methods still represent the gold standard in microbiological routine diagnostics. Some body fluids, such as blood, contain only limited pathogens (1–100 cfu/ml), which have to be enriched in blood cultures (BC)s first; other samples, such as stool or sputum, contain pathogens besides many other microorganisms that belong to the body flora and have to be separated to obtain pure microbial colonies for the diagnostics.

BC and agar plate-based diagnostics are suitable for most bacteria and fungi. These routine microbiological methods depend on the growth medium and the presence of specific substrates, antibiotics, or inhibitors, and can yield information about the species and the resistance phenotype, but without any information on the present serotype or genotype. Some pathogens are fastidious or require special culture techniques that are not routinely applied, so they can easily be overlooked.

The determination of viable bacteria via serial dilution, plating, and counting of the colony-forming units (cfu/ml) on specific agar plates is also a diagnostic approach which indicates the bacterial load that correlates with an

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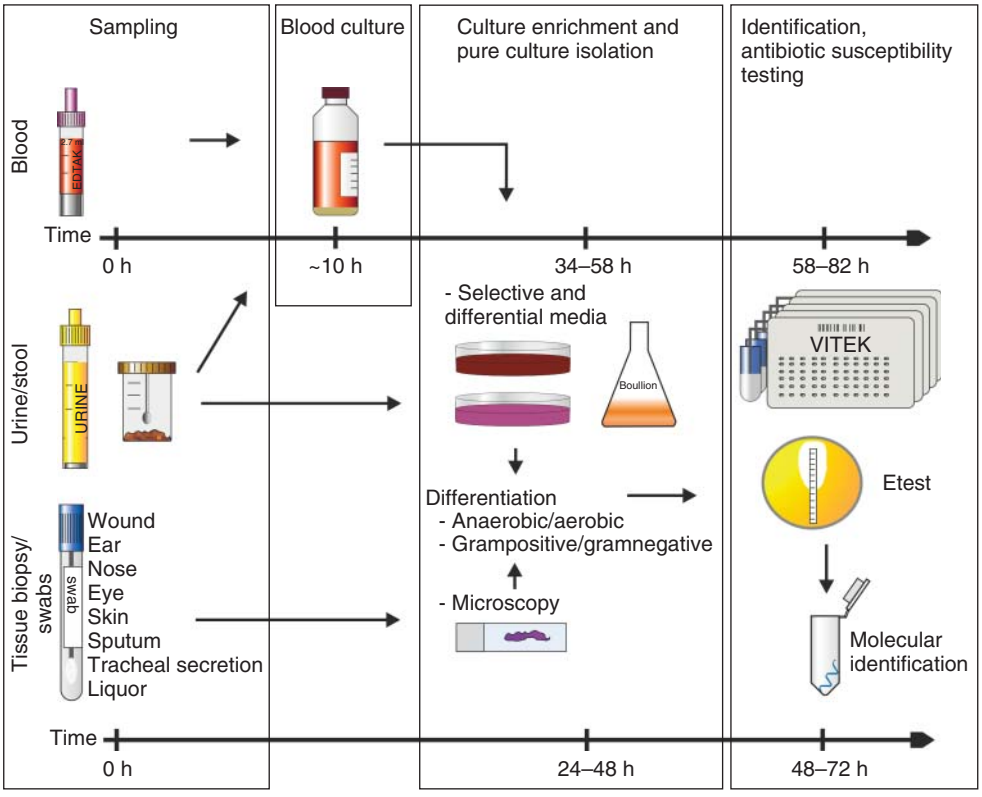

Figure 2.1 The workflow of culture-based diagnostics.

infection or colonization in some specimens such as urine or pharyngeal lavage.

Figure 2.1 presents a standard workflow and the timeline of a culture-based routine diagnostic.

2.3.1 Blood Culture

Several automated BC systems are presently on the market (e.g., BACTEC™, (BD), BacT/ALERT® (bioMérieux), or VersaTREK™ (Trek Diagnostics)), but the main principle involved is quite similar. Blood taken from a venipuncture is injected into two or more BC bottles that contain selective media for aerobic and anaerobic growth [10] (see also Figure 2.2). BC bottles containing media suitable for isolation of *Mycobacteria* or some fastidious fungi are also available, but they are usually used only when these pathogens are suspected.

The inoculated BCs are incubated under continuous monitoring of the growth, which depends on the system. For example, pH or gas pressure can be measured,

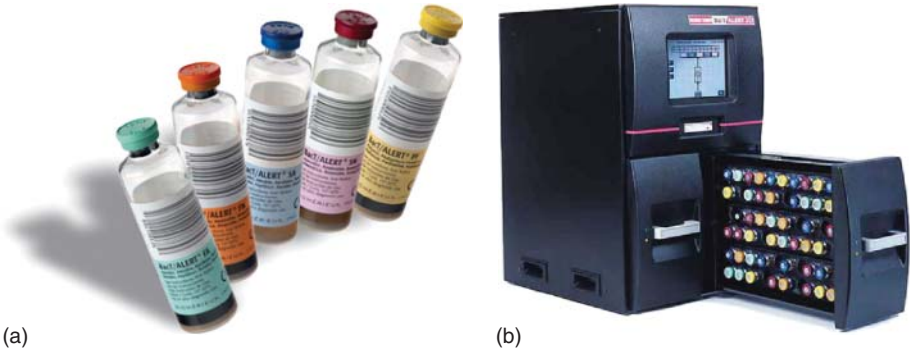

Figure 2.2 Blood culture bottles (a) and the automated blood culture module (b). Source: bioMérieux.

which yields a positive signal when the parameter achieves a defined threshold. This time to positivity (TTP) can take up to 48 h (or even weeks) for some species, but most cultures become positive within 24 h. It is one of the key parameters and hence the main disadvantage of BCs. Thus improved systems promise faster results within 10–15 h by optimizing the growth conditions of the media and increasing the detector sensitivity.

The fungal BC has been described to be less efficient in the determination of fungal infections, with most determined *Candida* spp. [11]. *Aspergillus* spp. infections were even more challenging with a median TTP of 8.5 days [12]. Presently, fungal BCs have improved in performance, but if bacterial BC is used, the cultures are almost always negative.

Some studies have shown that the common practice of testing only two or three BCs allows detection of only 90% of all blood-stream infections, so as many as four BCs may be needed to increase the sensitivity to >99% [13, 14]. Some other disadvantages of BC are (i) the risk of contamination by skin flora (e.g., *Staphylococcus epidermidis*) during the process of sample collection which leads to false-positive results; (ii) administered antibiotic therapy prolonging the TTP and often leading to false-negative results; and (iii) the inoculum effect, which might also yield false-negative results.

2.3.2 Automated Differentiation Systems

Once the microorganisms are separated, they can be analyzed by automated systems. Here, the most common commercial systems are Vitek®2 (bioMérieux, Inc.) (Figure 2.3a), Phoenix™ (Becton Dickinson and Company) (Figure 2.3b), or MicroScan (Siemens) [15]. These systems allow the identification of the species and antimicrobial susceptibility testing by using the corresponding assays.

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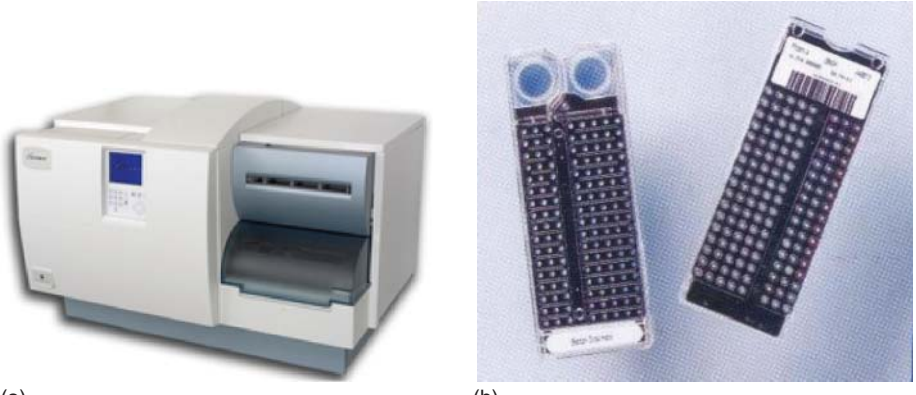

(a) (b)

Figure 2.3 Automated systems and devices for species and resistance identification. Source bioMérieux (a) and BD Phoenix (b).

Species differentiation of all systems is based on the determination of specific metabolic products or the ability to grow on specific substrates. These systems offer suitable "cards" or panels for Gram-negatives, Gram-positives, yeast and yeast-like organisms, aerobic or spore-forming bacilli (*Bacillaceae*), and also for some fastidious species. The cavities of these ID panels contain all supplements, and thus the microorganisms are applied as NaCl suspensions and are incubated, depending on the system and assay, between 10 and 48 h (or even longer). The results of the cavities are measured by colorimetry and/or turbidity, and they are compared with the database to identify the pathogen. Thus, a trained assistant has first to decide which identification assay has to be applied on the basis of sample's origin and the colony's morphology. Use of the wrong assay will yield unexpected results which usually cannot be validated by the system.

Susceptibility testing is usually performed on a second panel simultaneously with the ID panel. A variety of antibiotic susceptibility panels are available, including only those antibiotics that are related to the particular pathogens. Contrary to BD Phoenix or the MicroScan systems, the Vitek®2 systems do not perform a microdilution testing for all antibiotics. Here, only some concentrations are included within the empirically known MIC (minimal inhibitory concentration) range for the particular pathogen and antibiotic. The growth is measured by the turbidity and compared with a database. The algorithms that interpret the ID and resistance results depend on the information embedded in the particular database and the implemented expert rules (susceptibility guidelines), which are continuously adjusted to the resistance dynamics. Automated systems are presently quite accurate, but the implemented logarithms often are unable to handle uncommon resistance patterns, yielding false-positive or false-negative results that have to be further analyzed. Here, some discrepancies have been described mainly for *Enterobacteriaceae* and some β-lactam antibiotics, but also for some rare species.

For example, low-level resistance to cephalosporins, ureidopenicillins, and carbapenems can be overlooked. Moreover, misinterpretations can also occur when (i) multiple resistance mechanisms are present, such as enzymes (β-lactamases), efflux pumps, or porin loss; (ii) different β-lactamases are encoded; and (iii) the levels of expression of resistance determinants (enzymes or efflux) change. For a detailed review on automated systems and their performance, see [15].

2.3.3 API System

The analytical profile index (API) is a fast method to identify and classify microorganisms. It was introduced by Pierre Janin in the 1970s, and system is now manufactured by bioMérieux. For enterobacteria, it contains, for example, 20 cavities of miniaturized, dehydrated, conventional biochemical tests (e.g., citrate test, Voges–Proskauer reaction, galactosidase, etc.) which are inoculated with a microbial suspension. After incubation, the results can be read out manually or automatically. On the API20E strip (for *Enterobacteriaceae*), the tests are organized in groups of three, with corresponding values of 1, 2, or 4 as indicated for each well (Figure 2.4). By adding together the respective values of the positive tests, a seven-digit numeric profile is obtained which is compared with a database to identify the species. The coliforms have to be confirmed first by an oxidase test, which constitutes the 21st test with the value 4 if positive. Though initially the API20 was designed for *Enterobacteriaceae* (API20E), presently a variety of API stripes with increased tests and modified designs are available covering also fastidious species.

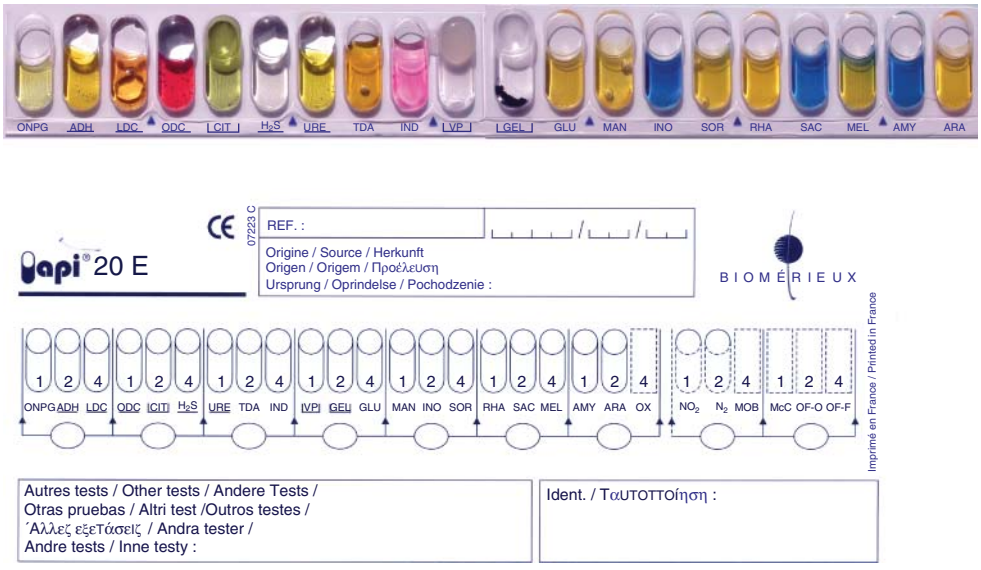
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Figure 2.4 API20E for species identification. Source: bioMérieux.

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2.3.4 Selective Agar

Bacteria and fungi can be directly preanalyzed or enriched on different selective or differential agar plates that contain specific substrates and indicators. The choice depends on patient's material and specimen's origin. Selective media are those that allow the growth of certain types of microorganisms while inhibiting the growth of others. Differential media contain specific dyes or indicators that allow the identification of closely related organisms. Commonly, both media types are combined.

For example, intestinal pathogens can be selected on eosin-methylene blue (EMB) agar, which allows the differentiation between bacteria that ferment lactose and those that do not, inhibiting the growth of Gram-positives. Because of the combination of the dye eosin and the pH indicator methylene blue, colonies of *E. coli* show a dark center and a greenish metallic sheen, whereas *Enterobacter aerogenes* colonies are pinkish and *Salmonella* spp. are uncolored. MacConkey and Hektoen enteric (HE) agars are also selective for Gram-negative bacteria with respect to lactose fermentation. Both media inhibit the growth of most Gram-positives by bile salts. MacConkey agar contains the dye crystal violet and a neutral pH red indicator. Here, colonies of lactose-fermenting species appear pink and those of nonfermenting species appear uncolored. HE agar contains bromophenol blue (pH indicator), acid fuchsin dye, sodium thiosulfate, and ferric ammonium citrate. Therefore, coliform species appear as orange colonies, *Shigella* spp. colonies green-colored with dark blue-green centers, and *Salmonella* spp. colonies blue-green without dark centers. Species producing H_2S show black-centered colonies.

Phenylethylene alcohol agar (PEA) inhibits the growth of most Gram-negative and swarming pathogens (such as *Proteus* spp.), thus it is used to select Gram-positive species such as *Streptococcus* and *Staphylococcus*. In combination with 5% sheep blood, it is selective for both Gram-negative and Gram-positive obligate anaerobe species. PEA is commonly used for purulent specimens or when mixed pathogens are suspected.

Mannitol salt agar (MSA) is used to select *Staphylococci* pathogens from mixed specimens. The high salt content (7.5%) inhibits the growth of other microorganisms. It contains mannitol as the carbon source and phenol red as pH indicator. *Staphylococcus aureus* ferments mannitol, yielding acid by-products that turn the pH indicator to yellow and thus can be distinguished from *S. epidermidis*.

Buffered charcoal yeast extract (BCYE) agar is primarily used for the selection and cultivation of *Legionella* spp. It contains L-cysteine to enhance the growth of *Legionella* spp. and activated charcoal to remove toxic metabolic products. For improving selectivity, various dyes and indicators may be added, as well as antibiotics that inhibit the growth of other species.

Agar-based methods tend toward rapid, extended chromogenic media that select and differentiate some species and simultaneously identify common and relevant resistance phenotypes (such as ESBL, carbapenemase, or MRSA) within

24–48h. Chromogenic media contain substrates that release a pigment or dye after enzymatic digestion. For example, coliform species can be visualized by indoxyl-β-D-galactoside (X-gal) because they produce a β-galactosidase which hydrolyzes the substrate by releasing the insoluble dye 5,5′-dibromo-4,4′-dichloro-indigo that accumulates in the cells and thus the colonies become blue pigmented.

2.3.5 Susceptibility Testing

Frequently used procedures for susceptibility testing are still broth microdilution, disk diffusion, the double disk test, or the Etest [16]. Microdilution is one of the earliest methods now miniaturized to a 96-well microtiter plate. The broth containing twofold dilution of the antibiotic (e.g., 1, 2, 4, 8, and 16 μg ml^{-1}) is inoculated with the standard 10^5 cfu/ml. After incubation for 17–24 h, the growth of the microorganism is determined by turbidity, and the lowest concentration that prevents growth represents the MIC. There are commercially available pre-prepared antibiotic panels as well as automated instruments that perform microdilution and measure the MIC (like the BD Phoenix panel). Since the dilution is not linear, MICs in between two higher steps (such as 16 and 32) are missed. However, the defined breakpoints (EUCAST or CLSI) are relatively low (e.g., for *Enterobacteriaceae* ceftazidime $S \leq 1$ and $R \geq 4$), and therefore it does not play a crucial role in diagnostics. More critical is the accuracy, which is considered to be ±1 dilution step for manually performed tests.

The disk diffusion method is performed on an agar plates (Mueller–Hinton agar) but is only qualitative without yielding an MIC. Here, the bacterial suspension is plated on the agar, and commercially prepared paper disks with fixed antibiotic concentrations are placed on the surface. After incubation for 16–24 h, the diameters of the growth-inhibition zones are measured (Figure 2.5b). The inhibition zone diameters are related to the susceptibility of the pathogen and the diffusion rate of a particular antibiotic through the agar. The inhibition zone diameters can be qualitatively interpreted by published guidelines and breakpoints (EUCAST or CLSI). There are many modifications to this method, such as the double disk synergy test (DDST). Here, two or more disks containing different antibiotics or inhibitors (e.g., EDTA) are placed at a short distance (e.g., 25 mm center to center), and the synergistic effects can be observed if the inhibition zone of one disk widens out in the direction of the other.

Antimicrobial gradient diffusion is the principle of the commercially available Etest (bioMérieux). It combines the qualitative agar diffusion method and the quantitative MIC determination. Here, plastic strips are impregnated with a dried antibiotic concentration gradient, which is indicated on the upper site. The strips are placed on agar plated with the pathogen suspension. After incubation, the MIC can be directly read form the strip as the lowest part of the ellipse-shaped inhibition zone (Figure 2.5a). This test is expensive but accurate, and is particularly used to verify results from automatic resistance testing.

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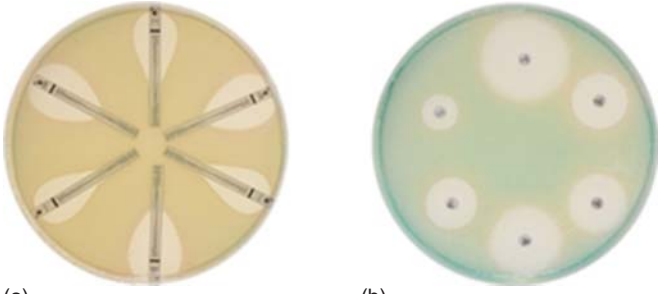

(a) (b)

Figure 2.5 Susceptibility tests. (a) E-test and (b) disk diffusion test. Source: bioMérieux.

2.3.6
Other Methods

Recently, two *in vitro* colorimetric tests for determination of β-lactamases with extended substrate spectrum (ESBL NDP [17] and Carba NP [18]) have been presented and recommended by the EUCAST. Both tests are based on the hydrolysis of the β-lactam ring by the corresponding cephalosporinases or carbapenemases and the color change of a pH indicator (phenol red) due to the hydrolytic products. They can be performed in less than 2 h but exhibit a sensitivity of $\sim 10^8$ cfu/ml. Thus, despite the strongly reduced time-to-result, this method is limited to a high cell density and thus needs pure cultures.

2.4
Nucleic Acid-Based Techniques

Diagnostic methods based on NATs are characterized by their improved sensitivity and reduced time effort compared to culture-based identification. However, the detection limits of some methods are still not suitable for direct analysis of patients' material because of the low pathogen content. Other fluids and samples, even when applying an adequate method, represent an intractable challenge for the analysis because of the high organic and inorganic contents (such as heme in blood or bile in stool) that interfere with PCR (polymerase chain reaction)-based applications. NAT, similar to ISH, benefits from the unique pattern of the genetic code and thus might be sufficient to determine almost any pathogen. Nowadays, most of the NAT-based methods are relatively cheap and can easily be established as routine protocols without the need for experts for execution and evaluation of the results. Here, some most common applications for routine usage, which represent only a few examples from the numerous possibilities of NAT-based methods, can be discussed. A detailed description of the broad spectrum of applications, as well as technical aspects of NAT, is dealt with in Chapter 3.

2.4.1 PCR

Analyses of the amplicons generated by enzymatic PCR are widely used in routine laboratories and can, in principle, be applied to detect all pathogens in many sample specimens. Prior to PCR analysis, the DNA has to be isolated from a pure or mixed culture, a single bacterial colony, a swap, or a tissue extract. Here, many commercial kits meeting the different requirements of the sample specimen are available, but also classical methods (e.g., phenol–chloroform–isoamylalcohol extraction) can be suitable.

First-generation PCR-based diagnostics suffer from unknown sequence data of adequate and discriminative targets that could provide a comprehensive coverage of the estimated pathogens. Presently, extensive sequence databases offer many discriminative targets that can be amplified by specific primers, and the limiting factor of many laboratories is that they cannot perform high-throughput analyses by the standard PCR-cycling instruments. Thus, the trend is to couple PCR to techniques such as mass spectrometry (MS) in fully automated procedures [19, 20].

2.4.1.1 Real-Time PCR

The introduction of the real-time polymerase chain reaction (RT-PCR) technique provided a highly sensitive and quantitative method to determine specific pathogenic contents. RT-PCR is similar to PCR but the amplified products are visualized in real time by fluorescent probing. Based on the principle used, one can distinguish between SYBR Green-based and FRET (Förster resonance energy transfer)-based methods. SYBR Green, which fluoresces when accumulates in any double-stranded DNA, provides high sensitivity but no specificity. Target-specific detection can be obtained by using fluorescent probes and the FRET principle. FRET is a mechanism that describes the energy transfer between two chemophores/fluorophores. Here, different techniques are common, with TaqMan as the most relevant. TaqMan probes are labeled by a donor fluorophore (5′, often the fluorescein derivate FAM) and an acceptor quencher (3′, often the rhodamine derivate TAMRA (tetramethyl rhodamine)) which annihilates the FAM fluorescence. During replication, the 5′-exonuclease activity of the *Taq*-polymerase releases the FAM, which can be detected, and accumulates with increasing PCR products. Molecular beacons and dual FRET hybridization probes can be also used, but since they are less common in diagnostics, they will not be dealt with here. (For a detailed review discussing the variety of FRET-based RT-PCR applications, see [21].)

RT-PCR allows fast confirmation of the presence of relevant genes encoding, for example, resistance mechanisms: *mecA* (methicillin resistance in staphylococci) or *vanA* and *vanB* (vancomycin resistance in enterococci); as well as fast adaptation when new variants occur (e.g., *mecC* an altered methicillin resistance gene with only 70% homology to *mecA* [22]). This method is also sufficient to distinguish single mutations, enabling discrimination of highly homologous variants.

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However, single-mutation analysis by RT-PCR is complicated and error-prone, and usually sequencing methods are preferred in this case.

One of the great benefits of RT-PCR is the possibility to do quantitative analysis. This is routinely used for the detection of viral nucleic acids in blood plasma to determine and monitor the viral load during antiviral therapy in transplanted patients: for example, determination of the EBNA-1 gene of EBV to control PTLD (posttransplant lymphoproliferative disorder) [23]. It is also used to predict the phase or course of other viral infection such as HIV, HSV, or CMV [21].

2.4.1.2 **Multiplex-PCR**

PCR protocols that are performed using more than two primer pairs yielding more than two PCR products are known as *multiplex-PCR*. This technique has incredibly increased the diagnostic capacity of PCR-based methods. Some laboratories have developed their own multiplex-PCRs to verify specific species or variants of a gene, which can be analyzed by the amplicon length on agarose gels [24], but commercial kits are also available that contain all necessary components including the primers to amplify a set of particular genes: for example, the RT-PCR-based assay, which simultaneously detects and differentiates the most important respiratory viral pathogens – influenza A, influenza B, and respiratory syncytial virus [25]. (A detailed review discussing the multiplex-PCR as diagnostic tool in virology can be found in Ref. [26].) However, multiplex-PCR (with up to hundreds of primers) is commonly performed prior to further specific downstream analyses such as probe hybridization in microarrays or MS analysis (see Chapters 3 and 4).

2.4.1.3 **RAPD and rep-PCR**

The RAPD (randomly amplified polymorphic DNA) technique is a PCR modification in which a single, short, and unspecific primer is used. This primer can anneal and prime at multiple locations throughout the genome, yielding specific amplicon patterns for different species and strains. Therefore, RAPD is often used for analysis of the relatedness between isolates in epidemiologic tracking. Here, also the rep-PCR has to be mentioned, which is performed using two primers that anneal to repetitive elements in the genome yielding, also, a specific amplicon pattern (in this case termed DNA fingerprint).

2.4.1.4 **Microarrays**

A microarray can be simply described as a two-dimensional microscopic collection of single features that can provide either quantitative (gene expression) or qualitative (diagnostic) data. Various types of microarrays exist, but most interesting for diagnostics are antibody microarrays (to be discussed in Section 2.5) and DNA microarrays. The DNA microarray technique is based on the hybridization of unknown nucleic acid targets to known probes which are attached to a solid matrix. The commercialization can be traced back to the paper in *Science* by Ron Davis and Pat Brown in 1995 [27]. Presently, the best known companies providing DNA microarrays are Affymetrix, Agilent, Arrayit, and Illumina, which offer fully designed arrays but also custom-specific printing or synthesis.

For diagnostic purpose, the probes are usually spotted (printed) on the matrix, resulting in 100–150 µm features with relatively low spot density. Other immobilization principles, such as *in situ* synthesis (>10^6 features per 1.2 cm^2), are more common for applications such as transcriptome analysis. The initially developed DNA microarray used radiolabeled targets [28], but presently fluorescence labeling is predominantly used.

To overcome the diversity problem of the analyzed genes and to increase the specificity, most systems use the multiplex amplification to label the amplicons prior to specific hybridization on a chip or slide (e.g., the DNA-microarray platform Check-Points [29] or the Verigene System, Nanoshere, Inc.). Details on the commercial microarray platforms, their performance, and diagnostic usage are given in Chapter 4. However, it should be mentioned that, compared to classical microbiological methods, this technique is still quite expensive, so it often presents only an additive approach [30].

2.4.1.5 RFLP

The method of restriction fragment length polymorphism (RFLP) rests on single mutations within specific restriction sites within the genome. In this method, the entire chromosome(s) is (are) digested by an endonuclease, and the restriction fragments are separated in an agarose gel. Here, pulse-field electrophoresis has to be applied because of the large DNA fragments. To increase the specificity, Southern blot might be performed after electrophoretic separation. This method is sometimes used for epidemiologically tracking microorganisms, but also to determine genetic alteration in some diseases. However, it is time consuming and laborious, so not routinely used for microbial diagnostics but used for epidemiologic issues.

2.4.2 Sequencing

Sequencing is the most accurate method to determine single mutations. Sequencing analysis can be performed from amplicons, or plasmidal or chromosomal DNA. For diagnostic purposes, mainly ribosomal RNA-encoding genes or conserved constitutive genes are used.

2.4.2.1 Ribosomal RNA Genes

To discriminate the pathogen, commonly the 16sRNA gene (for bacteria) or the 18sRNA gene (for fungi and other eukaryotes) is used. These genes contain variable and conserved regions that allow assigning the sequence to a genus or species (variable region) or to a subspecies (conserved region). However, these targets bear some risk of contamination. Since PCR and sequencing are highly sensitive, the exiguous content of foreign DNA (e.g., contamination of the solutions by microbes or free DNA) can be amplified by the 16sRNA or 18sRNA primers and interfere with the sequencing signals. Therefore, other conserved genes (like *gyrB*) are often used for analysis.

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Nucleic acids of viruses are also analyzed by sequencing to determine altered variants. In case of RNA from viruses, the RNA is reverse-transcripted to cDNA first.

2.4.2.2 MLST

MLST (multilocus sequence typing) is a highly discriminative typing system of most bacteria and some other organisms. Therein, internal regions of 450 – 500 bp of multiple housekeeping genes (usually seven) are sequenced and the differences within one gene of a given species are assigned as alleles. Each allele is assigned a unique allele number. In this manner, each analyzed strain is characterized by a set of (seven) allele numbers that represent a specific profile that can be assigned to a specific sequence type (also called the *MLST-type*, *http://www.mlst.net/*), allowing a fast comparison of the phylogenetic relatedness between the strains. This approach is usually not performed routinely in diagnostic laboratories, but in the case of a suspected endemic accumulation of a given pathogen, MLST and sequencing of further relevant genes such as resistance markers can be performed by national reference centers, thereby yielding a definitive discrimination.

2.4.2.3 NG Sequencing

These highly advanced sequencing techniques, collectively known as next-generation sequencing, are real-time methods exploring the release of luminescence (pyrosequencing in 454 Sequencing system of Roche Diagnostics), fluorescence (Illumina technology), or pH changes (IonTorrent by Life Technologies) when a nucleotide becomes incorporated during elongation of the target DNA. The range of applications offered by NG sequencing is very wide, including whole genome sequencing, transcriptome analysis, single nucleotide polymorphism, and so on. Some details concerning pyrosequencing will be also discussed in Chapter 3, but it has to be mentioned that this technique generates an impressive amount of sequence data in a relatively short time. This is a step closer to genome sequencing as a diagnostic tool. Presently, some techniques are available that differ more or less in the length of sequenced fragments, the detection principle, or the amount of generated data per run (IonTorrent, Illumina, SOLiD, 454), but none of these systems is presently used in the routine diagnostics.

2.5 Serology

Serologic methods are based on the very strong and specific antibody – antigen interaction. Antibodies are immunoglobulins (Ig's) that are produced by B cells and recognize and bind foreign objects, such as bacteria, viruses, or other particles and compounds, to induce their clearance by macrophages or by the liver or spleen. This section gives a brief introduction to Igs with the focus

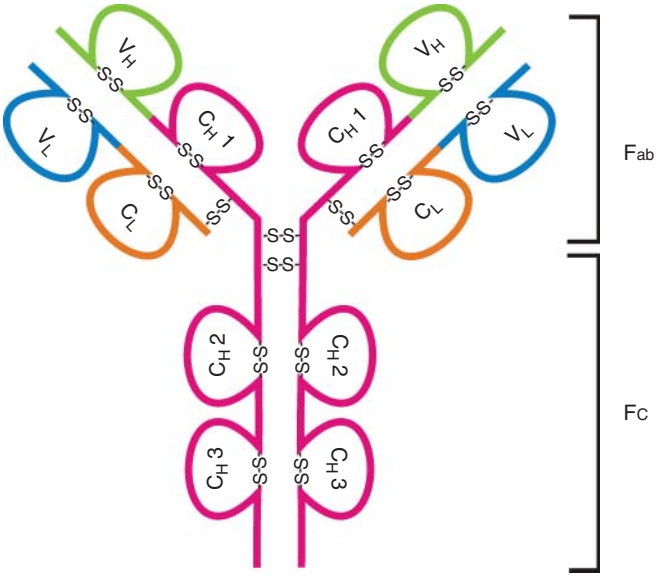

Figure 2.6 Structure of the IgG.

on antibody–antigen interaction as well as the aspects concerning diagnostic matters.

The main human Igs are IgA, IgG, IgE, and IgM; all are made of two identical heavy chains (H) and two identical light chains (L) which are covalently bound to form a Y-shaped protein. The light chain exhibits a variable domain (V_L) and a conserved domain (C_L); the heavy chain is made of one variable domain (H_V) and three (IgA, IgG) or four (IgE, IgM) conserved domains (C_H1, C_H2, C_H3, and C_H4). The variable domains of both chains form the antigen binding site, called the *complementary determining region* (*CDR*). The *antigen-binding fragment* (Fab) can be removed from the *crystallizable fragment* (Fc) by enzymatic digestion (e.g., by papain or pepsin) (for details see the Figure 2.6). The high variability of the CDR is randomly generated by genetic *V(D)J recombination* during the early stage of B cells and T cells. Thereby, IgM represents the predominant spontaneously produced antibody which appears also in the absence of apparent antigenic stimulation. These natural antibodies are produced by long-lived and self-renewing B1 cells, and the CDR region is polyreactive to phylogenetically related structures such as nucleic acids, polysaccharides, peptides, or phospholipids [31–33].

The natural IgM is a pentameric Ig that binds the invading pathogens with high avidity and activates the first-line defense via the complement system. It binds relatively weakly at the antigen but activates the complement system that is responsible for the pathogen clearance with high efficiency [34, 35]. IgM plays an essential role in the defense against bacterial and systemic infections [36] but also against viruses [37]. Its concentration decreases during the course of infection, and therefore the presence of IgM is always an indicator for a primary and acute infection. IgM stimulates further the production of IgG.

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IgG exhibits a high affinity to the antigen but activates the complement system less effectively than IgM. It is produced with a delay of approximately 3 weeks but has a prolonged average half-life of 21 days; in contrast, IgM has an average half-life of approximately 28 h [38]. IgG can be determined even years after a passed infection or antigen exposition by vaccination. The avidity of the IgG is initially low after primary infection but increases during the following weeks and months. During the secondary response, IgG exhibits a higher avidity compared to the primary response. This is due to the antigen-driven clonal expansion of high-affinity B cells.

2.5.1 Antigen–Antibody-Based Methods

The interaction between an antibody and the antigen is characterized by a high degree of affinity and very low dissociation constants. Antibodies can be divided into polyclonal and monoclonal probes, and antigens can be large (e.g., bacteria cells or viruses) or small (heptens). A monoclonal antibody recognizes a specific antigen epitope and is produced by one clonal B-cell line, whereas polyclonal antibodies are derived from plasma and represent a mixture of antibodies from different B-cell lines; thus these can recognize various AG epitopes. Complexes formed between monoclonal antibodies and their antigens or those formed between polyclonal antibodies and heptens are usually soluble. Polyclonal antibodies and large antigens form insoluble complexes. Moreover, antibodies have at least two antigen-binding sites (IgG, IgA, IgE, and in case of IgM, 10 antigen binding sites). Thus large aggregates of antibody–antigen complexes precipitate and can be determined in liquid media as well as in semisolid (gel) media by various methods.

2.5.1.1 Agglutination

Antibody–antigen complexes can be also visualized by the formation of large aggregates when carrier particles like polystyrene particles (such as latex) are involved, or by whole cells such as erythrocytes or bacteria cells. This process is named *agglutination* and is very sensitive and widely used in the diagnosis of bacterial infections and in blood typing of red blood cells. Agglutination tests are preferably performed using antibodies or antigens bound to latex beads, allowing a result within approximately 15 min. Moreover, this method is very simple, usually does not need highly purified samples, and is widely applicable and nonhazardous. The direct latex agglutination test is performed using antibodies bound to the beads to detect the presence of specific antigens in a biological sample, whereas the indirect test uses immobilized antigens to determine the presence of the corresponding antibody. A quantification of the antibodies or antigens can be performed using the inhibition mode. For example, a fixed quantity of the specific free antibody is mixed with a dilution of the test sample. The antigens form soluble complexes with the antibodies. Latex-immobilized antigens are added to the reaction agglutinating with the unbound antibodies.

The degree to which the antigen in the test sample inhibits the aggregation of the carrier particles indicates the concentration of antigen present in the sample. This principle can be reversed to determine the antibody concentration.

The main disadvantage of particle-based tests is the nonspecific agglutination due to the organic compounds in body fluids, which may inhibit or cause agglutination. Thus, sample pretreatment, such as enzymatic digestion, thermal denaturation, or strong dilution, might be necessary to decrease the sensitivity and accuracy of the assay [39]. Moreover, some agglutination assays are costly and thus not appropriate for routine diagnostics.

2.5.1.2 Immunodiffusion

There exist some immunodiffusion tests that are performed in agarose gels. Therein, holes are made in the gel and the sample of interest is applied in one of them (if more than two holes are used, then the sample is applied in the middle hole). The neighboring holes are filed with purified antibodies and the gel is incubated for approximately 48 h. The AG of the sample and the AB diffuse toward each other throughout the gel and become visible as opaque bands of precipitates in certain areas located between AB and AG. Tests using this principle are known as *Ouchterlony assays* [40] or *Oudin–Mancini tests* [41, 42], and have been widely used since their development in the mid-1940s. However, these methods have almost been replaced by the enzyme-linked immunosorbent assay (ELISA), which is more sensitive and suitable for quantification.

2.5.1.3 Quantitative Immunoassays

Radioimmunoassay (RIA) was the first highly sensitive quantitative immunoassay based on the antibody–antigen recognition that was developed in the 1960s [43] for the determination of very low concentrations of antigens (such as hormones, enzymes, or DNA). Here, a known quantity of radiolabeled (e.g., γ-radioactive iodine-125 isotope) antigens is mixed with a known quantity of antibodies. Then, a sample with unknown antigen quantity is added (e.g., blood plasma) that compete with the radiolabeled antigens (inhibition mode). Since commonly this method utilizes bead-immobilized antibodies that precipitate upon antigen recognition, the released radiolabeled antigens can be determined in the supernatant and the radioactive counts correspond to the concentration of the antigen of interest. Using known standards, a standard curve can be generated that allows the quantification of the unknown antigens in the sample. Alternatively, the decrease in the radioactive counts can be measured in the pellet or when the antibodies become immobilized, for example, in a microtiter plate well. An altered version of RIA is known as immunoradiometric assay (IRMA) in which a second radiolabeled antibody is used that binds at the first antigen-specific antibody.

Radiolabeled compounds require special precautions, licensing, and instruments. Thus, nonradioactive methods such ELISA, whose sensitivity is comparable to the that of RIA, has become more popular. The ELISA principle was developed by Engvall and Perlman, independently, in 1971 [44]. Here, the antibodies or antigens are linked to enzymes like POD, alkaline phosphatase

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(AP), or glucose-6-phosphate dehydrogenase (G6PDH). These enzymes hydrolyze chromogenic, fluorogenic, or chemiluminescent substrates, thereby releasing dyes or chemiluminescence that can be determined by colorimetry (absorbance), fluorescence, or luminescence. The miniaturization of the ELISA to a 96-well microtiter plate allows a higher throughput of the assay and reduced costs.

Corresponding to RIA, enzyme-labeled antigens are used in the competitive enzyme immunoassay (EIA) to determine the antigens of interest in complex samples such as blood plasma. In noncompetitive ELISA, two antigen-specific antibodies are used, of which one antibody is immobilized at the surface of the microtiter plate well in excess. When the antigen is bound, the second enzyme-linked antibody is added, which binds to the antigen to form an antibody–antigen–antibody complex (sandwich principle).

The antibodies or antigens can also be labeled by fluorophores (e.g., fluorescein), TAMRA derivatives, or luminescent substances (e.g., luminol). In this case, the methods are known as fluorescence immunoassay (FIA) or (electrochemi-) luminescence immunoassay (ECLIA, CLIA, or LIA). The sensitivities of FIA and LIA are comparable to that of RIA [45]. Moreover, these methods are faster than the conventional ELISA methods because the fluorescence or luminescence can be directly measured without any enzymatic reaction that has to be run prior to detection [46].

2.5.2 Automated Immunoassays

Some modified immunoassays are also available for automated systems nowadays, allowing faster analysis in a high-throughput manner in routine laboratories. Here, three methods are predominantly used: fluorescence polarization immunoassay (FPIA), microparticle enzyme immunoassay (MEIA), and chemiluminescence immunoassay (CMIA or ChLIA).

In FPIA, the phenomenon of fluorescence anisotropy is used when the excitation is performed with polarized light. (The principles of fluorescence anisotropy can be found elsewhere [47].) The changes in the polarization axes due to antigen–antibody interaction can be determined by special fluorescence spectrometers containing adjustable polarization filters. The preference of proteins to adhere to artificial surfaces is realized in MEIA. The greatly increased surface of the polystyrol microparticles (diameter about 0.5 μm) increases the reaction kinetics, allowing very short incubation times (compared to EIA or RIA). The microparticles are separated from the solvents by irreversible adhesion on a glass fiber matrix. The reaction is visualized by chemiluminescence: for example, dephosphorylation (AP) of 4-methyl-umbelliferylphosphate to 4-methyl-umbelliferone. This method can be used in the competitive and noncompetitive sandwich mode. CMIA uses paramagnetic particles instead of polystyrene, and therefore purification is performed using a magnetic device.

Several automated systems are commercially available: TDxFLx™, IMx™, AxSYM™, and ARCHITECT™ (Abbott Diagnostics), PersonalLab and Nextgen Four (Adaltis US Inc.), Avida Centaur (Bayer Health Care Diagnostics Division), Synchron LXi, UniCell Dxl 800 (Beckman Coulter Inc.), and so on. All offer different applications and techniques. (For a detailed overview, see Ref. [48] or visit the webpage *http://www.captodayonline.com/productguides/*.)

Luminescent oxygen channeling immunoassay (LOCI™, commercialized by Perkin-Elmer in the Alpha Technology Platform) uses two bead-bound antibodies (sandwich principle) where one bead is additionally labeled with a luminescent dye (e.g., olefine) and the other with a photosensitizer (phthalocyanine), which release a singlet oxygen ($^1O_2$) when excited by a specific wavelength. In the presence of the corresponding antigen, when both antibodies are bound, the proximity of the two types of beads leads to the transfer of the oxygen and to the emission of luminescence.

2.5.3 Applications of Serological Test

2.5.3.1 Types of Human Antibodies and What They Indicate?

The presence of specific antibodies in human specimens (such as blood plasma) indicates whether the person has been exposed to a particular pathogen or antigen. Here, the most relevant Igs for diagnostics are IgA, IgM, and IgG.

IgA is found in high concentrations in mucous membranes, such as respiratory and gastrointestinal tracts, saliva, and tears. It often indicates mucous membrane-associated infections but also autoimmune disorders. Moreover, IgA levels increase in response to the presence of allergens, so it is also used to determine allergenic antigens.

The presence of IgM indicates a primary and acute infection, whereas IgG can indicate an acute infection at a later stage or a secondary infection/reactivation (e.g., Herpes viruses such as HSV, CMV, or EBV). Avidity, which represents the relative averaged strength of the interactions between the antibody binding sites and their antigenic determinants, can be an additional marker for the infection's course enabling the differentiation between primary and secondary infection. Avidity increases with the infection's course and time. It is also an important parameter for the determination of the protective efficacy of vaccines [49]. The avidity test has significantly improved the serological diagnosis of many viral infections.

The infection's course can be followed by quantification (titer) of the particular antibody by repeating the analysis at day 10–14 after the first test. The titer is performed by a serial dilution of the blood plasma or other specimen; the higher the titer, the lower the reactive dilution. An increasing titer during the infection's course indicates that the infection is getting worse.

2.5.3.2 Validity of Antibodies against Opportunistic Species

The presence of antibodies against specific pathogens or antigens in blood plasma can be determined by using different methods as described above, and commercial

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kits are available for many pathogens. Immunoassays show usually very high sensitivities and specificities, but the sensitivity for some species is critical because many pathogens are opportunistic commensals of human body or exhibit a high prevalence (e.g., herpes viruses HSV, CMV, and EBV), and thus antibodies can be determined without any indication of infection. In such cases, it is of particular importance to observe the titer and to distinguish between IgM, IgG, and IgA.

2.5.3.3 Specificity of Fungal Antigens

Invasive fungal infections are difficult to diagnose using BC methods, and prolonged TTP has been shown in some cases with sensitivities that have been described to be less than 50% [50]. Therefore, serological methods determining fungal metabolites and antigens circulating in body fluids, such as blood and urine of BAL, have been developed. Here, mainly components of the fungal cell wall or antibodies against those are used for diagnostics: for example, mannan, galactomannan, and β-(1,3)-D-glucan.

Various studies have shown the suitable performance of these antigens. For mannan in *Candida* infections, sensitivities in the range of 69% and specificities >95 have been determined [51]. β-(1,3)-D-Glucan determination in fungal infections showed slightly worse results, with sensitivities around 60% and specificities between 79 and 97% [52]. The high negative predictive value of 75–100% helps in ruling out an invasive *Candida* infection, but the inconsistent positive predictive value of 59–96% complicates a clear diagnosis [51–53]. Galactomannan is found in many fungi (*Candida* spp., *Aspergillus* spp.) and thus is not strongly specific for a species. For aspergillosis, sensitivities of around 71% and specificities of around 89% have been reported [54]. Since galactomannan is also a common nutrient and drug additive, false positive results or cross reactions have been described for subjects on diets or drug therapy.

2.5.3.4 Specific Urine Antigen Tests

After the filtration of the blood in the kidneys, the plasma proteins largely remain in the blood, but to a less extent (1 : 1000) they can also be found in the urine. Smaller molecules can enrich to higher contents. Thus urine represents a kind of modified ultrafiltrate of the plasma [55]. This specimen can be obtained without any risk or invasive procedure from patients, and therefore the development of commercial diagnostic tools based on serological principles and urine as material is in great demand. Presently, some species can be determined in urine using commercial instruments, such as for the intracellular bacterial species *Legionella* spp. or *Mycobacterium tuberculosis*, or some fungi (*Cryptococcus gattii*, *Penicillium marneffei, Histoplasma capsulatum*).

2.5.4 Biomarkers

Biomarkers are molecular or physical indicators of health or illness or predictors of a risk of an illness or of the disease course, and thus are often divided into the

following subclasses: (i) diagnostic, (ii) prognostic, and (iii) predictive. Therefore, one biomarker can be meaningful for one or more subclasses. Biomarkers can have a qualitative or quantitative character and can further represent the so-called trait (invariable, such as genetic mutations) or state (e.g., varying concentration of specific enzymes or ions) markers. Circulating components, such as enzymes, sugars, organic acids, vitamins, ions, and hormones are used as biomarkers, but also DNA and RNA alterations (e.g., mutations, methylation, copy number) or protein modifications (e.g., phosphorylation, acetylation) can be used. The analysis can be made on various specimens such as blood, urine, liquor, tissue biopsies, or bone marrow. Depending on the biomarker of interest, different methods can be applied for detection and quantification.

Usually, biomarkers are significant for other noninfectious diseases (diabetes, arthritis, Alzheimer's disease, cardiovascular disorders, etc.). In the context of infectious diseases, only a few biomarkers can be reported to exhibit some predictive and diagnostic relevance. Here, mainly the procalcetonin (PCT) and C-reactive protein (CRP) levels are used to diagnose bacteremia. Both markers exhibit moderate sensitivities and specificities ranking between 60 and 90% depending on the study, specimens, and patient cohort [56–58].

2.6 Conclusions and Perspectives

Direct diagnostic methods such as microscopy have some benefits compared to other methods. They are faster than culture-based methods and can determine the infection before the patient has mounted a measurable antibody response that yields a reactive serologic test result. They also offer some additional information regarding tissue damage, intracellular pathogens, and parasites, and in some cases they are the most appropriate diagnostic tools for fastidious species (*Mycobacteria* spp., *Borrelia* spp.). However, the sensitivity and specificity of the staining methods strongly depend on the species and sample specimen, and many stains are not at all specific for the species. Therefore, these methods require experienced personnel and are often laboratory-intensive.

Culture-based methods are presently the gold standard in routine diagnostics, but are often insufficient in determining fastidious and slow-growing species. Moreover, parasites and viruses cannot be determined in BC or on agar plates. Also, these methods require trained personal and often laboratory-intensive controls. The automated systems have low sensitivity and specificity, and often are unable to interpret uncommon or unexpected phenotypes [59]. Finally, the tests are time consuming (72 h).

Serological methods are highly sensitive and specific and can be used for qualitative and quantitative diagnosis. They offer many simplified tests that can be performed without laboratory-intensive efforts, such as urine test strips or ELISA-based kits. Moreover, they yield information about the immune status and infection's course and can provide additional predictive values for risks and outcomes.

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However, some fungal antigens exhibit low species specificities, and suitable antigens are elusive for many opportunistic species.

NAT-based methods have also the potential to overcome the problems of low sensitivity and specificity, and they have become faster because of advanced techniques. Here, the main drawback is that only those pathogens and resistances can be found for which specific primers or probes have been used in the reaction or assay. In contrast, using culture-based or microscopic techniques, unexpected pathogens can also be found. Since the specificity and sensitivity of NAT-based features highly depend on the hybridization of the probes or the primers (annealing) and hybridization depends on the sequence homology, few mutational changes (such as substitutions, deletions, or inversions) of the target may impede the reaction and yield negative results. False-positive results due to contaminants are mainly a problem if highly conserved targets are amplified, such as ribosomal genes. But the observed trend of miniaturized and high-throughput techniques offers the possibility of combing an increasing number of relevant targets, allowing a micro-array-like comprehensive analysis of genes or gene transcripts. Thus the problem of missed or less specific targets can be overcome in future.

The NG-sequencing technology, which represents one of those miniaturized and high-throughput techniques, is presently fast developing, but still needs extensive computational capacity. Nevertheless, NG has the potential to reach the diagnostic routine in the near future and pave the way for the personalized medicine.

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3
Nucleic Acid Amplification Techniques

Marc Lehmann and Roland P.H. Schmitz

3.1
Introduction

Nucleic acid amplification techniques (NATs), more commonly but imprecisely called *polymerase chain reaction* (*PCR*), have evolved, among others, as central tools for pathogen identification and strain typing. The huge field of applications of the technique in all its modifications already marks the centerpiece of diagnostic approaches and has not only opened the door to a completely new understanding of elementary and sophisticated metabolic processes but also is the basis of the most important trends in life science industries. In a sense, it acts as the lens of a molecular microscope which visualizes the code from the genomic book, while its meanings have to be deciphered by a further armory of laboratory avenues.

In 1983, PCR was invented by Kary Mullis, for which he later (1993) was awarded with the Nobel Prize in Chemistry along with Michael Smith [1]. The protocol is based on targeting specific DNA or rDNA sequences with more or less specific short tracer sequences (primers/oligonucleotides, colloquially *oligos*) binding to flanking regions of the core sequence to be proliferated. A succession of thermal incubation steps defined by length and temperature are cycled and conduct double-stranded DNA (dsDNA) melting, that is, separation of the target DNA into antiparallel, complementary, single-stranded DNA (ssDNA) strands, specific binding of primers (annealing), and enzymatic amplification out of the basic set of four nucleotide monomers of the sequence embedded. In continuation, the newly synthesized dsDNA copies, selected in length by the specific primer binding sites (PBSs), themselves serve as templates for further replication rounds, setting in motion a chain reaction in which the DNA template is exponentially amplified. The core enzyme is a DNA polymerase, which was heat-sensitive at time of invention but subsequently was destroyed at the melting temperature. To gain useful numbers of target copies, an expensive and time-consuming replacement of the enzyme was needed at the beginning of each of the usually 20–40 replication cycles. The discovery of an extraordinarily thermostable DNA polymerase purified from the thermophilic bacterium *Thermophilus aquaticus*, which naturally lives in hot (50–80 °C) environments such as hot springs [2],

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paved the way for dramatic improvements of the PCR method. Mullis started to use the enzyme first in 1986, which was only needed to be added once at the beginning of the process, thus making the technique significantly more affordable and subject to automation, and opened the door for the upcoming revolutions in biochemistry, molecular biology, genetics, medicine, and forensics [3].

Applications were established in sequencing for genomic mapping/DNA-based phylogeny investigations, functional gene analysis, diagnosis of hereditary diseases (genetic predisposition), genetic fingerprinting in forensic sciences and paternity testing, detection of infectious pathogens, and selected antibiotic resistances. PCR technology facilitates the detection of DNA or RNA after reverse transcription into cDNA/copy DNA of pathogenic organisms and, as such, is the basis for a broad range of clinical diagnostic tests for various infectious agents, including bacteria, fungi, and viruses. PCR-based tests appear to have several advantages over traditional culture- and/or antibody-based diagnostic methods which measure the body's immune response to a pathogen and can detect the presence of pathogenic agents earlier than serological methods. Within the ("window") period between recent infection and immune response, such assays (e.g., enzyme-linked immunosorbent assays, ELISAs) are blind to their designated targets in affected donors unlike NAT-based tests, for example, for detecting early markers of HIV-1, hepatitis B, and hepatitis C infection, namely viral DNA or RNA, in blood.

In the following sections, we will focus on DNA polymerase-based and therefore actual PCR protocols, and later on workflows in which nucleic acids are proliferated using alternative enzymes in extraordinarily efficient but rather complex reactions, undermining the current license burden of the original procedure.

3.2 The Basic PCR Protocol

The PCR is commonly carried out in reaction volumes of 10–200 µl in small cavities (0.02–0.5 ml) in a thermal cycler. The PCR setup requires several components and reagents, which typically include the following:

- Template DNA (e.g., eukaryotic/microbial/viral), genomic, or mitochondrial (mtDNA), containing the target region to be amplified. For amplifying cDNA from RNA, reverse-transcriptase polymerase chain reaction (RT-PCR) is applied. The viral enzyme reverse transcriptase synthesizes a copy dsDNA (cDNA) from the RNA template, which is then amplified by PCR. Most PCR methods typically amplify DNA fragments of up to ~10 kb, although some techniques allow for amplification of fragments up to 40 kb in size [4];
- A set of two primers which are complementary to the three prime (3′) ends of each of the sense and antisense strand of the target sequence;
- Taq polymerase or another DNA polymerase with a temperature optimum at around 70 °C and stable at 95 °C for the length of cycling;

- 4 deoxynucleoside triphosphates (dNTPs);
- Buffer component (phosphate-based) providing a suitable chemical environment for optimum activity and stability of the DNA polymerase;
- Divalent cations, usually magnesium ions (Mg^{2+}) and manganese (Mn^{2+}), often used for PCR-mediated DNA mutagenesis, given that manganese increases the error rate during DNA synthesis as polymerase cofactors [5]. Lower concentrations of Mg^{2+} will increase replication fidelity, while higher concentrations will introduce more mutations;
- Potassium ions for modulation of the stringency of the reaction;
- Addition of reagents, such as formamide, in buffer systems, which may increase the specificity and yield of the reaction [6]. Denaturants (e.g., DMSO) can increase amplification specificity by destabilizing nonspecific primer binding. Other chemicals, such as glycerol, are stabilizers of polymerase activity. Detergents (such as Triton X-100) can prevent the polymerase from sticking to itself or to the walls of the reaction tube.

DNA polymerases occasionally incorporate mismatch bases into the extending strand. High-fidelity PCR employs enzymes with 3′–5′ exonuclease activity that decreases this rate of misincorporation (e.g., Pfu polymerase, isolated from the archeon ***P****yrococcus* ***fu****riosus*, displays proofreading activity, and a five- to eightfold decrease in the error rate of polymerization compared to Taq [7]). Since errors increase as PCR progresses, Pfu is the preferred polymerase when products are to be individually cloned for sequencing or expression.

The thermal cycler heats and cools the reaction tubes to achieve the temperatures required at each step of the process. This is usually achieved by Peltier elements which allow both heating and cooling of the block holding the PCR tubes simply by reversing the electric current. Thin-walled reaction tubes permit maximum thermal conductivity to allow rapid thermal equilibration (low ramping times). Heated lids prevent liquids condensing on top of the tubes and altering the concentration ratios within, formerly prevented, for example, by mineral oil or wax top layers over the reaction solutions. Extremely reduced ramping rates in rapid-PCR combined with ultrathin-walled reaction tubes allow significantly reduced cycle lengths and full PCR runs of less than 10 min under optimal conditions using a SpeedCycler^2, which achieves rates of up to 15 °C s^{-1} (Analytik Jena, Jena, Germany).

The temperatures used and the length of time they are applied in each cycle depend on certain parameters. These include the enzyme used for DNA synthesis, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (T_m) of the primers [8]. It should be noted that also constant (isothermal) temperature protocols exist, namely in helicase-dependent amplification, in which a DNA helicase, a DNA unwinding enzyme, is used in place of thermal denaturation [9] or the single-primer isothermal amplification (SPIA® technology by NuGEN Technologies Inc., Bemmel, The Netherlands).

However, the following cycle steps are usually programmed (the durations depicted are typically applied in standard PCR cyclers) (see also Figures 3.1 and 3.2):

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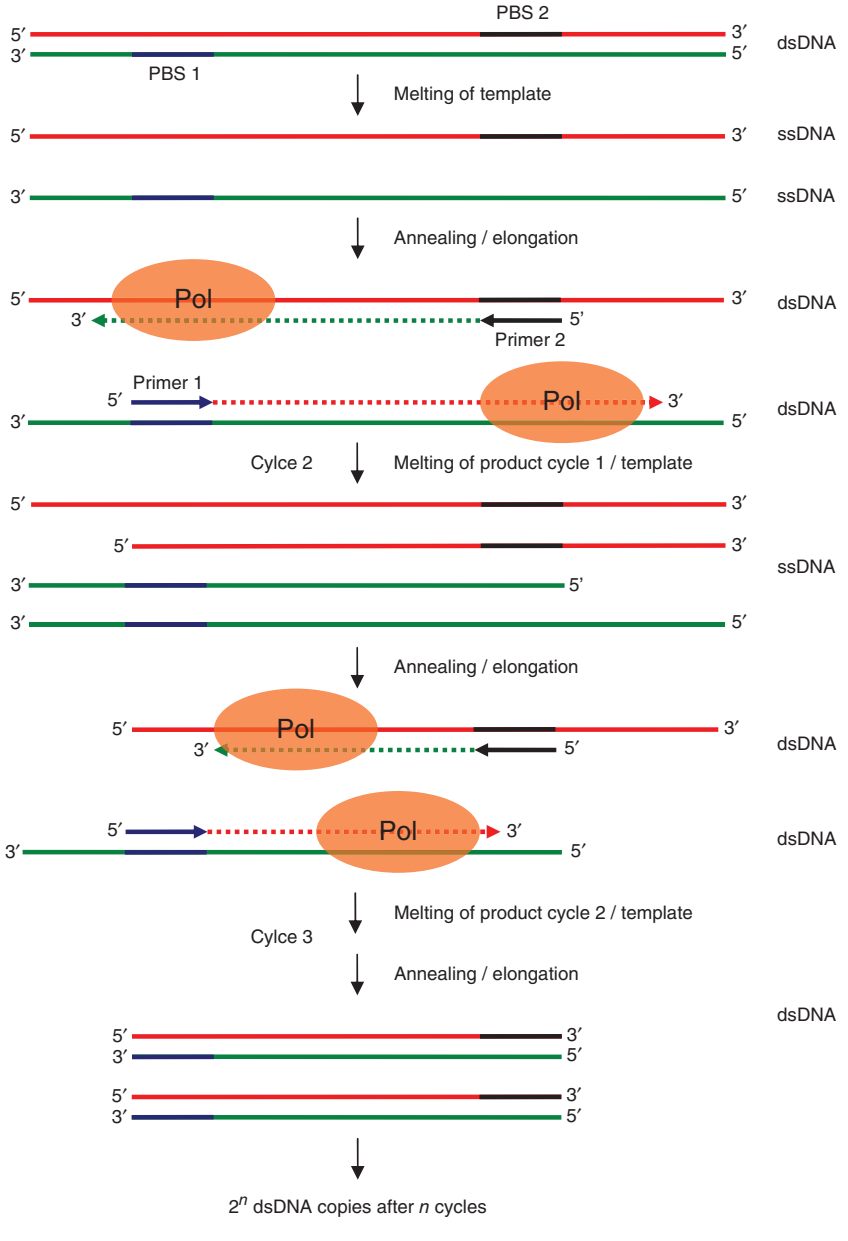

Figure 3.1 Scheme of PCR. For explanations refer to text. PBS: primer binding site and Pol: polymerase (e.g., Taq polymerase).

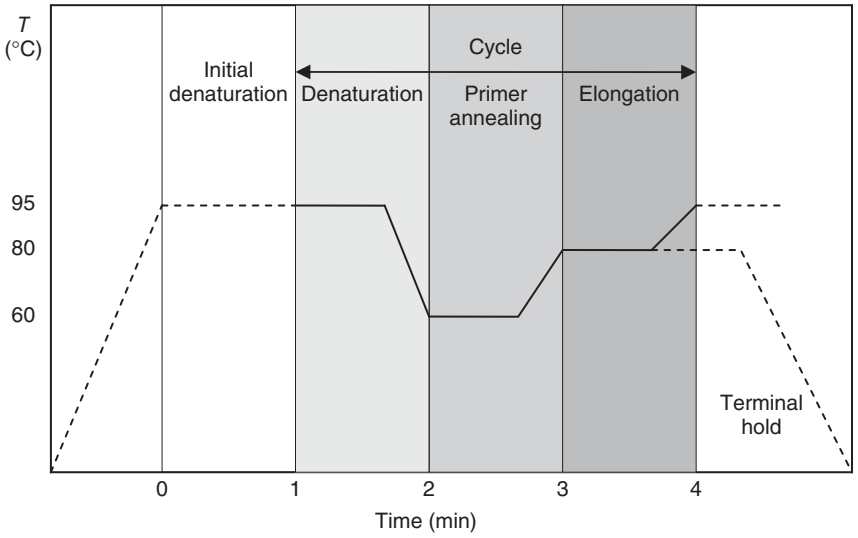

Figure 3.2 PCR temperature profile. For explanations refer to text.

- *Initialization*: Needed for *Hot-Start PCR*, as a modification of the conventional protocol, which reduces nonspecific product amplification during the initial set-up stages of the PCR. In this procedure, amplification cannot occur until the actual temperature is above that where nonspecific annealing of primers occurs. A hybrid DNA polymerase is used that is inactive at ambient temperature. Polymerase activity is inhibited either by binding of antibodies [10] or by the presence of covalently bound effectors that dissociate only after an activation step: the vials are heated up to 94–96 °C or even 98 °C, if extremely thermostable polymerases are used, for 10–15 min. Meanwhile some blocked enzymes are commercially available for which a special heat-activation step is not necessary. Prolonged high initial temperatures are also often applied for performing *Colony PCR*, where bacterial colonies are screened directly, sampled with sterile pipette tips, and transferred into a PCR mix. To release the DNA from the cells, the PCR is either started with an extended time at 95 °C when standard polymerase is used, or with a shortened denaturation step at 100 °C and a special chimeric DNA polymerase [11].
- *Denaturation*: DNA melting, that is, thermal cleavage of hydrogen bonds between the bases, at 94–98 °C for 20–30 s.
- *Annealing*: Binding of primers to ssDNA template at 50–65 °C for 20–40 s. The primer annealing temperature (T_a) usually is about 3–5°C below the median primer melting point (T_m) and is the estimate of the DNA–DNA hybrid stability: a too high T_a ($T_{a\,opt} = 0.3 \times T_m$ (primer) + $0.7T_m$ (product) − 14.9) will produce insufficient primer–template hybridization, resulting in low PCR product yield; too low may possibly lead to nonspecific products caused by a high number of base-pair mismatches. Mismatch tolerance is found to have the strongest influence on PCR specificity. Stringency of the reaction/stability

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of hybridization is further mainly influenced by salt concentration. *Touchdown PCR* may help to reduce nonspecific background by gradually lowering the annealing temperature as PCR cycling progresses. The annealing temperature at the initial cycles is usually 3–5 °C above the T_m of the primers used, while at the later cycles it is similar or below. The higher temperatures give greater specificity for primer binding, and the lower temperatures permit more efficient amplification from the specific products formed during the initial cycles, which increases sensitivity [12, 5].

- *Extension/elongation*: Enzymatic synthesis of a new DNA strand complementary to the DNA template strand by adding dNTPs in the 5′–3′ direction, condensing the 5′-phosphate group of the dNTPs with the 3′-hydroxyl group at the end of the nascent DNA strand. The temperature is highly enzyme-dependent with a Taq polymerase optimum at 75–80 °C. Extension velocity (by approximation 1000 bp min^{-1} at T_{opt}) depends on the DNA polymerase used. The length of the DNA fragment defines the required duration of the amplification interval. Under optimum conditions, that is, with no limitations with regard to substrates or reagents, exponential amplification is achieved by each cycle with only minute quantities of DNA needed to be present (several femtograms). The reaction usually decreases (leveling off) when substrates are incrementally limited and stops/reaches a plateau if they are fully exhausted (end-point PCR). Taq polymerase can amplify targets of over 50 kb [4].
- *Final elongation*: Optional process after last cycle to achieve a maximum consumption of nucleotides, performed at 70–74 °C for 5–15 min.
- *Final hold*: Optional indefinite short-term storage of reaction vial at 4–8 °C.

3.3 Primer Design

Crucial to achieve accurate NAT results is the design of the primers to be used. Counterpoints in primer design are as follows:

- *Primer length* is decisive for annealing and specificity. Optimal are 18–22 bp. However, in *Miniprimer PCR*, a thermostable polymerase extends the growing strand from primers as short as 9–10 nucleotides, a method which permits PCR targeting to smaller primer binding regions and used to amplify conserved DNA sequences, for example, 16S (or eukaryotic 18S) rRNA genes [13].
- *Amplicon length*: Dictated by experimental goals. For qPCR (quantitative polymerase chain reaction), the target length is below 500 bp optimal down to close to 100 bp; for standard (end-point) PCR, it is 100–500 bp, but >500 bp is also feasible.
- *Meltingtemperature (T_m)* of *primer/target hybrid*: temperature at which 50% of dsDNA hybrid has been divided into complementary ssDNA strands which mainly depends on the GC (guanine-cytosine nucleotide) content of the sequence (see also under melting curve analysis below). Primers with melting temperatures in the range 52–58 °C generally produce the best results, and

melting temperatures >65 °C tend to secondary annealing. The two primers of a primer pair should have closely matched melting temperatures for maximizing the PCR product yield. A difference of 5 °C or more can lead to failing amplification.

- *GC content*: The GC content of primers should be 40–60%. Presence of G (guanine) or C (cytosine) bases within the last five bases from the 3′ end of primers (*GC clamp*) promotes specific binding at the 3′ end because of the stronger bonding of G and C bases via three hydrogen bonds each (in comparison to A and T which hybridize via two hydrogen bonds). More than three G's or C's should be avoided in the last five bases at the 3′ end of the primer: an unstable 3′ end (less negative Gibbs free energy, ΔG) will result in less false priming. A stable 5′ end (more negative ΔG) will result in more efficient and specific bonding to the template.
- *Primer secondary structures*: Presence of secondary structures produced by inter and/or intramolecular interactions can lead to reduced reaction efficiencies by adversely affecting annealing and thus amplification. In general, hairpin structures should be avoided. Hairpins at the 3′ end with a ΔG of $-2\,\text{kcal mol}^{-1}$ and internal hairpins with a ΔG of $-3\,\text{kcal mol}^{-1}$ are tolerated ($\Delta G = \Delta H - T\Delta S$, where ΔH is the change in the enthalpy of the system, T is the temperature in degrees kelvin, and ΔS is the change in entropy). Self-dimers, formed by intermolecular interactions between two (same sense) primers where the primer is homologous to itself, reduce the product yield. A 3′-end self-dimer with a ΔG of $-5\,\text{kcal mol}^{-1}$ and an internal self-dimer with a ΔG of $-6\,\text{kcal mol}^{-1}$ are tolerated generally. Primer cross dimers are formed by intermolecular interaction between sense and antisense primers, where they are homologous. 3′ end cross dimers with a ΔG of $-5\,\text{kcal mol}^{-1}$ and an internal cross dimer with a ΔG of $-6\,\text{kcal mol}^{-1}$ are tolerated.
- *Repeats and runs*: Dinucleotide repeats occurring many times consecutively should be avoided for the reason of mispriming. The maximum number of dinucleotide repeats acceptable in an oligonucleotide is 4. Primers with long runs of a single base should generally be avoided for the same reason. The maximum number of runs accepted is 4 bp.

The sequence of the ssDNA target that may also fold into secondary structures has also to be considered. The stability of these template secondary structures depends largely on their free energy and melting temperatures. If primers are designed on secondary structures which are stable even above the annealing temperature, the primers are unable to bind to the template and the yield of PCR product is significantly affected. Regions of homology have to be avoided to improve primer specificity.

Normally, the primers used in PCR are designed to be fully complementary to the target. However, the polymerase is tolerant to mismatches away from the 3′ end. Tailed-primers include noncomplementary sequences at their 5′ ends. A common procedure is the use of linker-primers, which ultimately place restriction sites at the ends of the PCR products (amplicons), facilitating their later insertion into cloning vectors. Primers can also be designed to be "degenerate" using

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multiple nucleotides at particular ("Wobble") positions, able to initiate replication from a large number of target locations. The use of the nucleoside analog inosine, for example, allows the hybridization to three of the four regular bases.

Standard PCR can easily be modified to yield labeled primers for hybridization, for example, incorporating biotin-dUTP (biotin-labeled deoxyuridine triphosphate), radioactive or fluorescent dNTPs. Labeling might be done during or after amplification and can be combined with "asymmetric-PCR" (see the following text).

Irrespective of which type of primer is used, they are designed with regard to their grade of sequence conservation. Conserved sequences are particular similar or identical sequences maintained by evolution despite speciation. Highly conserved DNA sequences are thought to have functional value. Consequently, among the most highly conserved sequences are active sites of enzymes, binding sites of protein receptors, and those found within components involved in basic cell functions, for example, DNA polymerization or mRNA translation. Mutations in highly conserved regions mostly lead to nonviable life forms. Consequently, sequence similarities serve as evidence for structural and functional conservation, as well as of evolutionary relationships between the sequences. Although still no clear consensus exists on the degree of homology required to assign a specific genus or species, comparative analysis is the primary means by which functional elements are identified and this has usually been done to identify optimal sequences to derive primers and probes.

However, validation of assays for species detection which rely on specific primers has mostly been done with bacterial type strains. A type strain is the nomenclatural type of a species or subspecies, a "prototype strain," fully recognized as a member of the taxon species level. Other strains of the species have to share a common ancestor and a high level of similarity with it. Whenever possible, the type of a species or a subspecies should be a strain of proven clinical significance.

The type strain is made up of living cultures of an organism, which are descended from a strain designated as the nomenclatural type. This strain should have been maintained in pure culture and should agree closely to its characters with those in the original description [14]. Since August 2002, "authors of papers in which new names and/or new combinations are proposed to provide evidence that types are deposited in two publicly accessible recognized culture collections in two different countries (e.g., ATCC (American Type Culture Collection, Manassas, USA), and the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany)." This also guarantees the availability of the respective strain all over the world.

Use of those conserved regions within type strains therefore should result in a location-independent traceability of the target organisms by commercial assay target panels. However, due to evolutionary mechanisms, a mutational alteration of target sequences, which empirically mostly leads to nonviables, cannot be excluded entirely. This is particularly due to the fact that bacteria have the highest reproduction number/lowest generation time of all living organisms.

In consequence, some clinical strains isolated may not be traceable because of such mutational changes, even if degenerated primers are used. The problem increases with the breadth of specific primer combinations in multiplex assays, which cover also rather infrequent pathogens.

Primer review via public online tools (e.g., BLAST basic local alignment search tool, National Center for Biotechnology Information, NCBI, *http://www.ncbi.nlm.nih.gov/*) is recommended to reduce the risk of nonspecific amplification. Both the nucleotide sequence of the target and the primer sequence itself can be searched (e.g., download of target sequence under nucleotide NCBI; sequence alignment via clustal programs, *http://www.clustal.org/*). Numerous open primer design tools are available that can assist in PCR primer design and may reduce the cost and time involved in experimentation by lowering the chances of failed experimentation. Open access programs are, for example, CODEHOPs (COnsensus-DEgenerate Hybrid Oligonucleotide Primers, *http://bioinformatics.weizmann.ac.il/blocks/help/CODEHOP/CODEHOP_program.html*), FastPCR (*http://primerdigital.com/*), and Primer-BLAST (*http://www.ncbi.nlm.nih.gov/tools/primer-blast/*, NCBI) C, whereas AlleleID®/Beacon and Array Designer® (Premier Biosoft, Palo Alto, CA, USA), which can design primers and oligonucleotide probes for complex detection assays such as multiplex assays and cross-species primer design to reduce the cost of experimentation, are commercially available.

CODEHOP designs a pool of primers containing all possible 11 or 12-mers for the 3′ degenerate core region and having the most probable nucleotide predicted for each position in the 5′ nondegenerate clamp region. FastPCR, for example, assists in designing standard, long distance, inverse, multiplex PCR, group-specific (common primers for phylogenetically related DNA sequences), unique primers (for each from phylogenetically related DNA sequences) as well as, for *in silico* preparation of applications such as overlap extension polymerase chain reaction (OE-PCR) and primers used in degenerated PCR. The software utilizes combinations of normal and degenerated primers for melting temperature calculation based on the nearest neighbor thermodynamic parameters, and oligonucleotides can be designed for microarray analyses. Primers and probes are analyzed for secondary structures including hairpins, self-dimers, and cross-dimers in primer pairs.

PrimerPlex® (Premier Biosoft, Palo Alto, CA, USA) is a software that can design *Allele Specific Primer Extension* (ASPE) primers and capture probes for multiplex *Single-Nucleotide Polymorphisms* (SNPs) *Genotyping* using suspension array systems such as Luminex xMAP® (Luminex, Oosterhout, The Netherlands; see the following text) and BioRad Bioplex (Bio-Rad Laboratories GmbH, München; Germany). Computer simulations of theoretical PCR results (electronic polymerase chain reaction/ePCR, NCBI) may be performed to assist in primer design.

3.3.1 Specific versus Broad-Range Primers and the Fate of False Targeting

Besides applications relying on species- and/or genera-specific primers, universal or broad-range oligos are used in several approaches combining amplicon

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verification via probing or sequencing. However, the assays using universal primers are in part compromised by their exquisite sensitivities because of the fact that the addressed targets are often present in multiple copies (e.g., 16S or 23S rDNA targeting). Such oligos have proven to be suitable for nucleic acid trace detection in diagnostic consumables [15–18], contaminated diagnostics [18] or disinfectants, antiseptics, and blood culture media [19], or introduced via applying routine sample withdrawal techniques causing false-positive results.

Generally, contamination with nontargeted RNA/DNA should be addressed in lab protocols and procedures that separate pre-PCR mixtures from reaction batches. This usually involves spatial separation of pre-analytic processes with raw sample materials from analytic working places for amplification and/or purification of PCR products. Substantial hygiene measures are a prerequisite for contamination-free NAT testing, but they will be discussed elsewhere. Of further concern are clinically irrelevant results of nondisease-associated transient bacteremia, the presence of free bacterial or fungal nucleic acids due to translocation from native colonized surfaces, as well as detection of nonviable/nonactive pathogen cells. Circulation of translocated microbial DNA or the transient presence of bacteria in blood not associated with the disease have been proven *in vivo* [20]. Culture-positive bacteremia was reported after tooth brushing and other dental measures as transient translocations of mucosal colonizing microorganisms due to minimal manipulations already three decades ago and have been reproduced thereafter [21–23]. Moreover, isolation of bacteria in asymptomatic blood donors and even transient fungemia without apparent clinical significance has been repeatedly reported [24, 25]. Pure microbial background DNA has been detected in the blood of healthy individuals [26, 27] and asymptomatic blood cell donors [24]. This indicates the range of risks to obtain false positives and the difficulties to distinguish them from true positives. However, the origin and clinical significance of "false-positive" samples are often ambiguous and might belong to the yet-known host–pathogen interactions [28]. In conclusion, nonspecific PCR protocols promise to facilitate the finding of microorganisms that are found less frequently, even yet unknown, or generally noncultivable [29, 30] and might therefore be of high scientific relevance but rather objectionable in causative species verification.

The panel of multiplex PCR assays with specific primers is limited because of primer interactions/heteromer formation, which sets margins to the number of targets detectable within one PCR tube (primer pool). Otherwise, an array of too many individual PCR reactions for investigation of a single specimen would lead to high costs and workload. Such an approach would additionally lead to a target dilution and, therefore, reduced sensitivity. It has been consequently proposed that PCR detection in clinical diagnostics should focus only on those pathogens or resistance determinants that are not covered by guideline-recommended treatment regimens and that have been identified as the major cause of inappropriate treatment [31]. The authors stated that multiplex panels should be scaled down by high conservation of its individual primers. Such a narrower assay would be more cost effective, may achieve higher accuracy due to reduced intra-test interference,

Table 3.1 Pros and Cons in using broad range/universal primers (BRP) (vs specific primers) for pathogen detection in complex samples by PCR techniques.

Pros BRP	Cons BRP
High sensitivity	Frequent false-positives due to contaminating DNA detection after usage of, for example, contaminated consumables, inadequate sampling techniques
Detection of microorganisms that are found less frequently, even yet unknown, or generally noncultivable	Detection of nondisease-associated transient bacteremia
Finding of pathogens of current/local (clinical) importance	Overlayed datasets hamper final analysis
Nonrestricted target panel (continuously expanding data bank)	Often (misleading) indication of apparent multi-infection
Assays with low plex-grades possible (single-/oligoplex)	
Unlikely exclusion of targets by out-of-date or inadequate primer design	

and would better address current and emerging clinical needs. However, clinicians rightly call for all-embracing diagnostic approaches: what would be the benefit of an assay that gives only an aperture of the targets of relevance and necessitates continuation of the traditional clinical course in parallel to cover all diagnostic outcomes? Casually, the appropriateness of guided anti-infective measures has to be re-evaluated in any case in the future in accordance with the fact that current guidelines rest upon in part deficient diagnostics.

Concluding, the use of species-specific primers should be consciously balanced against carefully validated broad-range PCR protocols (see Table 3.1). Currently, it remains a question of philosophy to decide for a single- or multiplex panel, broad-range, or species-specific primers. The usability of an analytic approach has at any rate to be validated individually depending on the addressed sample material and the specific demands and must also cover the downstream analytic process.

Technical problems also arise by presence of more than one PCR amplicon, for example, after bacterial multi-infections or contamination, or due to sequence heterogeneities on different intra-genomic copies of the respective target gene [32]: overlayed sequences and therefore noninterpretable results with direct sequencing techniques are obtained frequently, a problem which is increased by the use of broad-range primers. Admittedly, this problem has been successfully addressed using broad-range dual priming oligonucleotides (DPO™) (Kommedal *et al.* [33], see also below under Section 3.10). Furthermore, iSentio (Paradis, Norway) offers research use only (RUO) online software solutions (e.g., RipSeq® Mixed) to optimize sequence-based identification from both single and poly-microbial samples. By the use of advanced algorithms, the need for manual isolation and recultivation of colonies from poly-microbial samples prior to 16S

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rDNA sequencing is restricted, which makes direct sequencing relevant for a broader range of clinical samples (e.g., abscesses and pleural fluids) [34].

3.4 Modified End-Point PCR Protocols

The basic NAT protocol has been modified for various applications, mainly for diagnostic purposes. Although some of the above-mentioned techniques are well advanced in years, they retain their basic potential and are still of interest. For example, the combination of several primer pairs within one reaction vial is often used to simultaneously address different target sequences (*Multiplex PCR*). This permits the simultaneous analysis of multiple targets in a single sample. In testing for diverse genetic mutations or DNA fingerprinting, oligoplex assays combining groups of three or four primer pairs are often used. In blood stream infection (BSI) pathogen diagnostics, more than 20 pairs are combined to address the multitude of potential pathogens (see the following text) (Tables 3.2 and 3.3).

Asymmetric PCR is used in some sequencing methods and hybridization probing to generate an overrun of one ssDNA strand as a product. PCR is carried out as usual, but with an excess of the primer for the strand targeted for hybridization. Because of the slow (arithmetic) amplification later in the reaction after the limiting primer has been used up, extra cycles of PCR are required [35]. A usual relation of both primers is 1 : 10 [36] and more. This method has been modified into linear-after-the-exponential-polymerase chain reaction (LATE-PCR), in which a limiting primer with a higher T_m than the excess primer is used to maintain reaction efficiency as the limiting primer concentration decreases mid-reaction [37].

Nested PCR is used to increase the specificity of DNA amplification by using two sets of primers in two successive reactions. In the first PCR, one pair of primers is used to generate a first DNA product, which may contain by-products amplified from nontarget areas. The desired main product from the first PCR is then used as the template of a second PCR, using one ("hemi-nested") or two ("semi-nested") different primers whose binding sites are located at the 3′ of each of the primers used in the first set.

Allele-specific PCR was used to detect SNPs (single nucleotide polymorphisms). It requires prior knowledge of a DNA sequence, including differences between alleles, and uses primers whose 3′ ends encompass the single-base difference. PCR amplification under stringent conditions is much less efficient in the presence of a mismatch between template and primer, so successful amplification with an SNP-specific primer signals the presence of the specific SNP in a sequence [38].

RT-PCR (see above mentioned) is widely used, for example, in expression profiling, to determine the expression of a gene or to identify the sequence of a viral RNA, including transcription start and termination sites. If the genomic DNA sequence of a gene is known, RT-PCR can be used to map the location of exons and introns in the gene. The 5′ end of a gene (corresponding to the transcription start site) is typically identified by *RACE-PCR* (*rapid amplification of cDNA ends-polymerase chain reaction*).

Table 3.2 Pros and Cons in NAT routine application in detection of sepsis pathogens.

Pros NAT	Cons NAT
BC positivity rate <10% in clinical routine; NAT positivity presumed to be sugnificantly higher	No information on most antimicrobial susceptibility patterns (only available for a small selection including mecA, vanA/B, and a few β-lactamases)
BC time-to-result ≫24 h versus <8 h for NAT	No information on expression status after detection of resistance targets
High sensitivity (down to 3 cfu/ml whole blood)	Lower sensitivity ≥ 3 cfu/ml whole blood (but assumed higher genome/target copies)
Higher number of targets (genome copies vs viable cells) than BC	High workload (depending on assay system)
High risk of BC contamination (~20%)	High hands-on time
Only detection of viable cells by BC	High running costs/initial investment for devices/lab accommodation
Detection of genomic targets of lyzed/nonviable/phagocytozed pathogen cells	No replacement of BC → additional costs/work load
Detection of fastidious/nonculturable/slow-growing microbes (e.g., fungi) in time possible	Clinical significance of targeting lyzed/nonviable/phagocytozed pathogen cells still under discussion
	Experienced personnel required
	Sensitivity for free/phagocytozed (microbial) DNA not defined
	Restricted target panel achieved with specific primers (only detection of the most prevalent pathogens)
	Only restricted dataset on patients' benefits available to date
	Higher risk of contamination due to complex workload/need for DNA-free reagents

BC: blood culture; cfu: colony forming units.

Methylation-specific PCR (MSP) is used to detect the methylation of CpG (cytidylate-phosphate-deoxyguanylate) islands in genomic DNA and therefore obtains information on the expression status of target genes [39]. DNA is initially modified by sodium bisulfite, converting all unmethylated, but not methylated, cytosines to uracil, and subsequently amplified with primers able to distinguish the modifications.

Several further modifications of standard PCR have been implemented for advanced, specialized, and routine applications as listed and discussed with respect for applications in bloodstream infection by Schrenzel [28]. They may in part significantly alter and improve the outcome of the protocol; the NAT itself will only be as suitable as the associated total DNA release and isolation protocols (to be discussed below under Section 3.9.3).

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Table 3.3 Current methods for sepsis pathogen detection in different protocols.

	Blood culture including ID/AST	SeptiFast (Roche)	Sepsitest (Molzym)	Prove-it Sepsis (Mobidiag/ Inverness) (post-culture system)	Hyplex® tests (AMPLEX) (post-culture system)!	Magicplex™ Sepsis Real-time Test (Seegene)	VYOO® (Analytik Jena)
Target panel bacteria (species)	Not limited	19	Not limitated (>345 bacterial and fungal species)	>50 species Identification of 27 bacterial species on species level, 30 more on taxon level	>10 species	73 Gram (+) bacteria; 12 Gram (−) bacteria	34
Target panel fungi (species)	Not limited	6	Only *Candida* spp.	—	—	6	7
Target panel resistances	Not limited	mecA	n/a	mecA	mecA	mecA, vanA, vanB	mecA, vanA, vanB, blaCTX-M15, blaSHV
Time-to-result	>24 h	<6 h (including mecA resistance)	7–8 h	3 h after positive blood culture	2–4 h after positive blood culture	>4 h	6.5 h

			~4 h until end of PCR plus sequencing				
Sensitivity/ specificity	~1 cfu/ml	3–30 cfu/ml	n.s.	n.s.	n.s.	n.s.	<10 to <100 cfu/ml
Sample volume	~10 ml	2 × 1.5 ml	2 × 1 ml	n/a	n/a	<0.5 ml	5 ml
Amplification/ result interpretation	Turbidity measure-ment/ID/AST	qPCR/ melting curve analysis	Standard broad-range PCR/ sequencing	Multiplex PCR/microarray	Standard multiplex PCR/ELISA	Standard PCR/qPCR/ melting curve analysis	Standard multiplex PCR/microarray
Sample material	EDTA and citrate whole blood	EDTA whole blood	EDTA and citrate whole blood	Isolated DNA	Isolated DNA	Whole blood	EDTA whole blood
Sample preparation	n/a	Mechanical cell lysis, total DNA isolation	MolYsis® – selective cell lysis, human DNA degradation, DNA isolation	n/a	n/a	Magicplex™ automatic extraction system SeePrep12™ (automated cell lysis and DNA isolation)	Mechanical cell lysis, magnetic bead-based DNA isolation, microbial DNA enrichment

ID/AST: microbial typing/antibiotic susceptibility testing; n.s.: not specified; and n/a: not applicable; EDTA: ethylenediaminetetraacetic acid.

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3.5 Non-PCR NAT: Isothermal Amplification Protocols

The purchase of worldwide PCR licenses by Hoffmann-La Roche in 1991 was the impulse to circumvent the patent restrictions and to develop alternative amplification strategies that do not depend on a (DNA) polymerase reaction. In succession, NAT methods like *SDA (strand displacement amplification)*, *NASBA (nucleic acid sequence based amplification)*, or *TMA (transcription-mediated amplification)* were introduced, with, in the mean time, a high market share of TMA-based systems developed by Gen-Probe (Gen-Probe Incorporated, San Diego, CA, USA), patented in the United States in 1995. Commercially available are, by now, high-grade diagnostic applications, for example, for the detection of HIV (human immunodeficiency virus), hepatitis B/C, HPV (human papilloma virus), *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*, with TIGRIS® and PANTHER® devices as fully automated Gen-Probe solutions.

TMA is based on RNA and DNA amplification, while DNA polymerase in PCR only proliferates DNA – or even RNA after reverse transcription of RNA into cDNA – in isothermal (but also cycled) reactions at typically 41 °C. The end-products per cycle are 100 – 1000 RNA copies. RNA – DNA and DNA – DNA hybrids are synthesized intermediately by action of two thermolabile enzymes, a reverse transcriptase with inherent DNA polymerase and RNase H activities, and an RNA polymerase [40]. Briefly, the method acts as follows (see Figure 3.3):

1) The first primer (bifunctionally exhibiting a T7 RNA polymerase promoter at the nonhybridizing 5′ end) hybridizes at the 3′ end of the target (sense) RNA template and the reverse transcriptase synthesizes the opposite complementary DNA strand, and a RNA – DNA heteroduplex is formed.
2) The endonucleolytic RNase H function of the reverse transcriptase selectively degrades the RNA template strand within the RNA – DNA hybrid (does not degrade ssRNA). Subsequently, the second primer attaches to the 5′ end of the remaining new synthesized cDNA strand with the RNA polymerase promoter sequence, and the DNA polymerase activity of the reverse transcriptase produces a complementary DNA (dsDNA).
3) The cyclic part of the protocol starts with binding of the T7 RNA polymerase triggered by the embedded promoter sequence. The enzyme produces antisense RNA copies from the dsDNA, without melting of the dsDNA prior to polymerization. The antisense RNA serves as template for binding of primer 2, which previously has bound to the newly synthesized (antisense) cDNA strand of the DNA – RNA hybrid. The process is the same as followed for the original sense strand. Further RNA – DNA hybrids are formed by action of the first primer and processed (as stated under step 2). The action sequences forming new dsDNA molecules starting from the sense and antisense RNA strands are different, but they are considered to be kinetically identical. More than 10^{10} copies can be generated in a period of merely 15 – 30 min.
4) Amplicon detection is accomplished by hybridization with (antisense) ssRNA-specific labeled probes.

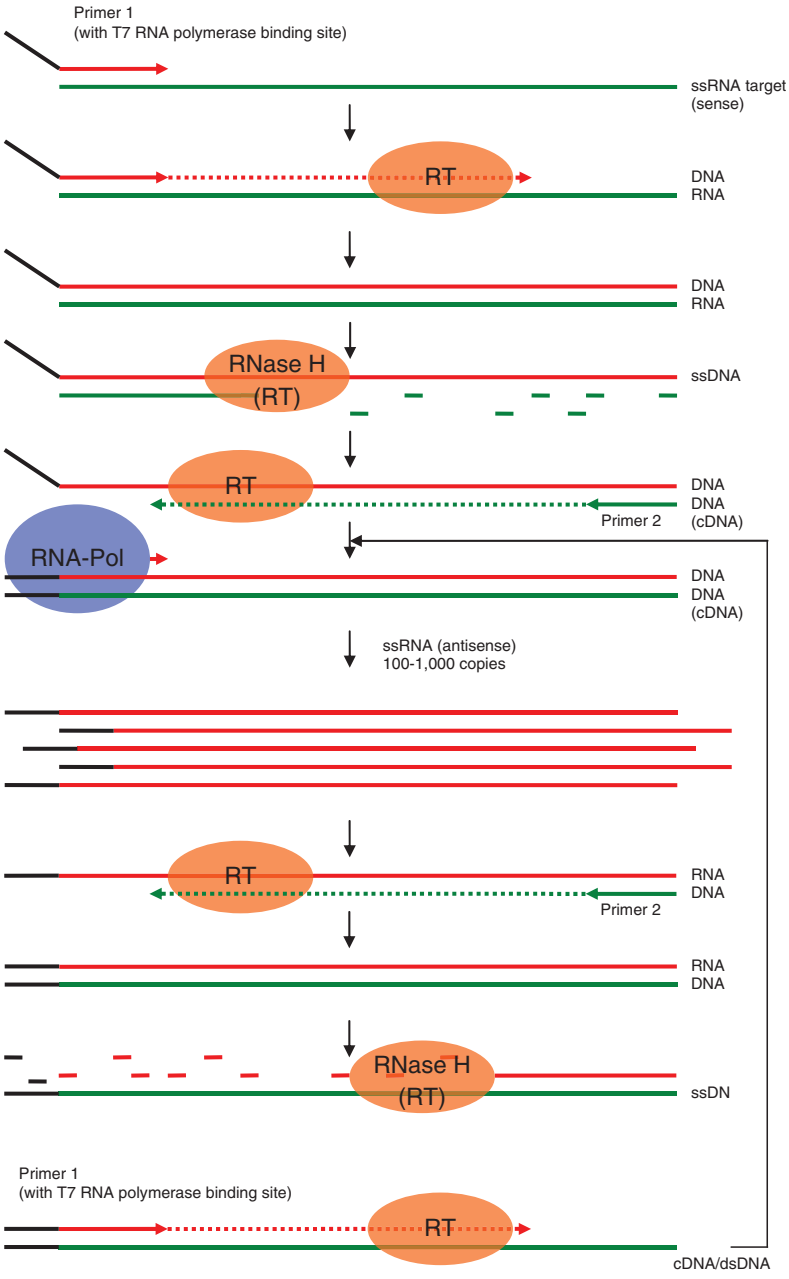

Figure 3.3 Scheme of isothermal *transcription mediated amplification* (*TMA*) of RNA. RNA–DNA and DNA–DNA hybrids are synthesized intermediately by action of reverse transcriptase (RT, with inherent DNA polymerase and RNase H activities) and RNA polymerase (RNA-Pol). Amplicon detection is accomplished by hybridization with (antisense) ssRNA-specific labeled probes. Modified after [40].

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NASBA [41] is a variation of the TMA method and increasingly used for cyclic RNA amplification, for example, for rapid diagnosis and quantification of HIV-1 in serum [42, 43]. The technique has been used to develop rapid diagnostic tests for several pathogenic viruses with single-stranded RNA genomes [44] and parasites like *Trypanosoma brucei* [45] and has been subjected to miniaturized assay solutions [46].

Loop-mediated isothermal amplification (LAMP) is an isothermal technique for the amplification of DNA [47] and requires a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops. Because LAMP recognizes the target by initially six distinct sequences and afterwards by four distinct sequences, it is expected to amplify the target sequence with high selectivity. Detection of the amplification product can be achieved photometrically or even visually because of an increase of turbidity by incremental manganese pyrophosphate in solution [48, 49].

While LAMP is widely used for detecting infectious germs, for example, in tuberculosis [50], malaria [51], and sleeping sickness [52, 53] in developing countries, it has potential for further applications. For whole-genome and whole-transcriptome amplification, a modification called *single primer isothermal amplification* (SPIA® technology by NuGEN Technologies Inc., Bemmel, The Netherlands; see the following text) was presented using DNA/RNA chimeric primers, DNA polymerase, and RNase at constant temperature. After the generation of a dsDNA molecule (template) with an RNA/DNA heteroduplex tag at one end, amplification is accomplished by binding and extension of a single SPIA primer. Initially, RNase H unmasks the priming site by digesting the RNA part of the tags, revealing an ssDNA sequence that is complementary to the SPIA primer. The primer binds to this site and is extended by a strand-displacing DNA polymerase to copy the complementary strand. The continuous amplification reaction gains up to microgram amounts of dsDNA (cDNA) maintaining the stoichiometry of input material, and can be applied for whole transcriptome, whole genome, or targeted sequence amplification.

A further approach is conducted by Hologic (*Invader® Plus Chemistry*, Madison, WI, USA) which utilizes two simultaneous isothermal reactions in a biplex format. The two-step sequential process improves the start-to-finish time for assays by combining PCR-based target amplification with subsequent signal amplification. After standard amplification of the target, heat denaturation of Taq polymerase precedes the Invader® reaction (10 min, 99 °C). Following this, a probe and an Invader® oligo anneal to adjacent sequences, generating an overlapping structure at 63 °C. A thermostable cleavage enzyme cuts off the overlapping part of the probe, releasing a 5′ flap plus one nucleotide. These steps are repeated successively, generating growing numbers of released flaps relative to the amount of target in the sample and allowing for quantitative detection of targets. The cleaved flaps combined with a FRET (*fluorescence (Förster) resonance energy transfer*) probe in a secondary simultaneous cleavage reaction generates a

fluorescent signal. Again, this process is simultaneously iterated, and causes an increasingly amplified fluorescent signal. The two simultaneous reactions typically produce a 1–10 million-fold signal amplification during a 1–4 h reaction. A fluorescence plate reader is used for readout of final results.

3.6 Quantitative PCR

qPCR (real-time PCR) methods allow the quantification of the amount of a given sequence present in a sample (accumulation of DNA product after each round of amplification), a technique often applied to quantitatively determine the levels of gene expression. It is also used for pathogen-targeting because of its extraordinarily high sensitivity (see the following text) and enables both detection and quantification as absolute copy number or relative amount (i.e., when normalized to DNA input or other genes). Amplified DNA is detected as reaction progresses in real time, typically measured by a fluorimetric real-time PCR instrument. Two alternative staining techniques are widely applied:

- Nonspecific fluorescent dyes (e.g., SYBR® Green or EvaGreen®) intercalate with any dsDNA and quantitatively within new synthesized amplicons. Product increase during PCR therefore leads to an increase in fluorescence intensity and is measured at each cycle, thus allowing DNA concentrations to be quantified (see melting curve analysis and high-resolution melting (HRM) below) (see Figure 3.4a). However, binding to nonspecific PCR products (such as primer dimers) can potentially interfere with, or prevent, accurate quantification of the intended target sequence.
- Sequence-specific DNA probes hybridize specifically with melted target sequences. Therefore, use of the reporter probe significantly increases specificity and enables quantification even in the presence of nonspecific DNA by-products. The methods rely on two basically different probe designs: (i) Corresponding hybridization probes labeled with one out of two different fluorescent dyes each (a donor and an acceptor moiety, respectively) bind to specific target sequences in close proximity to each other in a head-to-tail arrangement. Under this condition, excitation of the donor via FRET (fluorescence resonance energy transfer) [54] allows the excitation of the acceptor unit via light emission. Product increase during PCR leads to a rise in fluorescence intensity (see Figure 3.4b). (ii) Bifunctional DNA probes are equipped with a fluorescent reporter at one end and a quencher of fluorescence at the opposite end. The quencher dye molecule in close proximity of the reporter quenches the fluorescence emitted by the fluorophore when excited by the cycler's light source via FRET (see Figure 3.5). If the quencher moiety is a nonfluorescent black hole chromophore which emits the energy it receives from the fluorophore as heat, the probe is unable to fluoresce. Breakdown of the probe ("hydrolysis probes") by the 5′ to 3′ exonuclease activity of the Taq polymerase breaks the reporter–quencher proximity and thus allows unquenched emission

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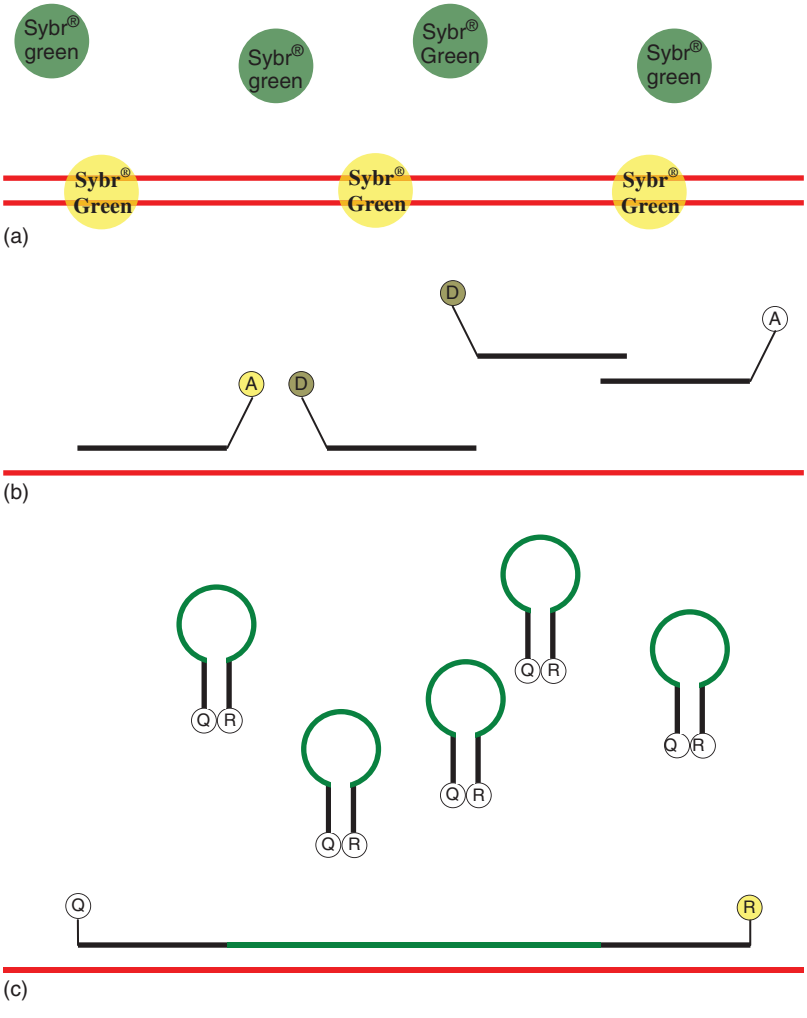

Figure 3.4 Modes of reporting I. The non-specific fluorescent dye *SYBR® Green* intercalates into dsDNA (a). Product increase during PCR leads to a rise in fluorescence intensity. B/C: Generation and quenching of fluorescent signals by *fluorescence resonance energy transfer* (*FRET*). (b) Corresponding *hybridization probes* labeled with two different fluorescent dyes (a donor and an acceptor moiety, respectively) bind to specific target sequences in close proximity to each other in a head to tail arrangement. Under this condition, excitation of the donor by the fluorescence reader allows excitation of the acceptor unit via light emission from the donor moiety. (c) Using *molecular beacons*, unfolding of the paired stem when binding to specific targets keeps reporting (R) and quenching (Q) moieties apart from each other and light emission occurs.

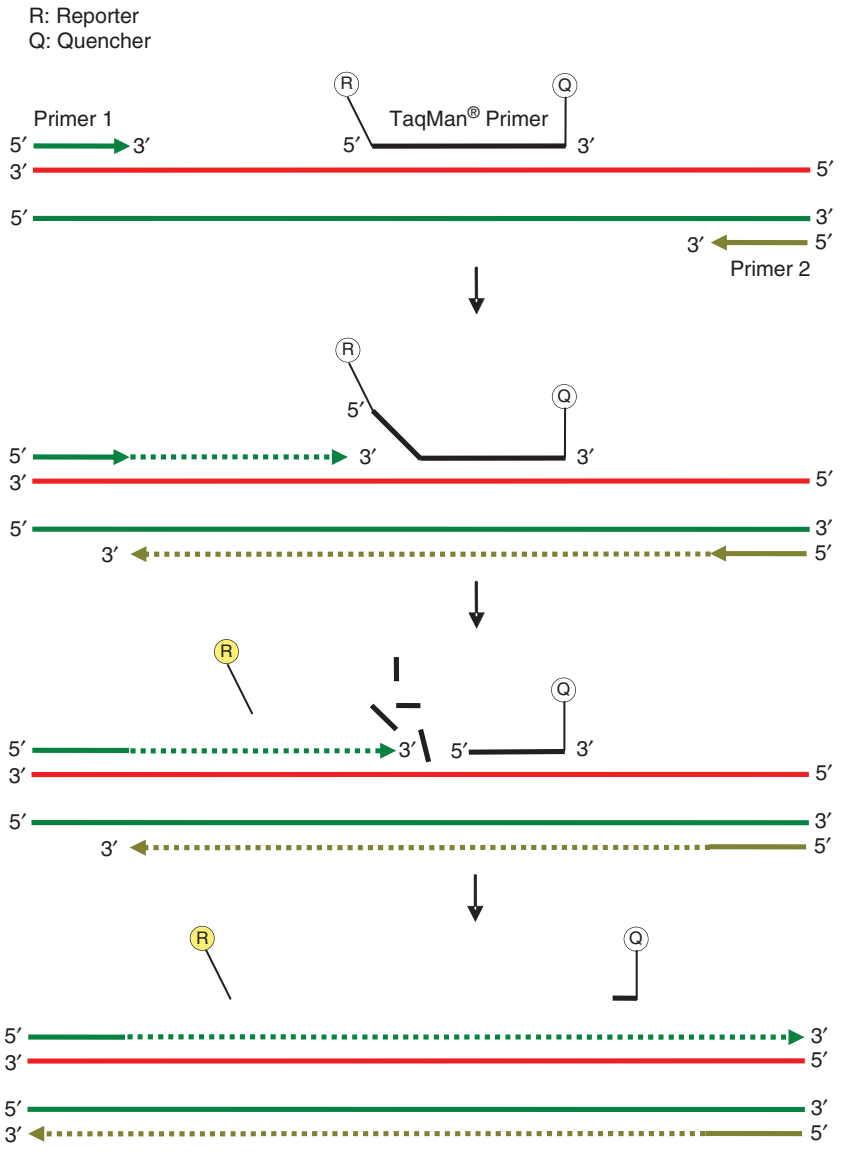

Figure 3.5 Modes of reporting II. Dual-labeled probes (e.g., TaqMan®) with a fluorescent reporter (R) at one end and a quencher (Q) of fluorescence at the opposite end will be degraded by Taq polymerase 5′ to 3′ exonuclease activity while anti-sense strand elongation. Breakdown prevents the reporter–quencher proximity and thus allows unquenched emission of fluorescence.

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of fluorescence, which can be detected after excitation. Hence, fluorescence detected in the real-time PCR thermal cycler is directly proportional to the fluorescence released and the amount of DNA template present in the PCR. An increase in the product targeted by the reporter probe at each PCR cycle therefore causes a proportional increase in fluorescence. Different colored fluorophores can be used in multiplex assays for the simultaneous detection of several genes, provided that all targeted genes are amplified with similar efficiency. However, fluorescent reporter probes do not prevent the inhibitory effect of primer dimers, which may depress accumulation of the desired products in the reaction.

TaqMan® probes are widely used in this respect, first reported in 1991 [55] and subsequently developed by Roche Molecular Diagnostics (Mannheim, Germany) for diagnostic assays and by Applied Biosystems (now Life Technologies, Darmstadt, Germany) for research applications. Several different fluorophores (e.g., 6-carboxyfluorescein (FAM) or tetrachlorofluorescein (TET)) and quenchers (e.g., tetramethylrhodamine (TAMRA) or dihydrocyclopyrroloindole tripeptide minor groove binder (MGB or DPI3)) are available [56].

Molecular beacons [57] are related to TaqMan® with regard to their basic probe structure (see Figure 3.4c). They are hairpin-shaped oligonucleotides with the loop portion acting as a probe sequence complementary to a target nucleic acid molecule. The stem is formed by annealing complementary arm sequences on the ends of the probe sequence. A fluorescent moiety is attached to the end of one arm, and a quenching moiety is attached to the end of the other arm. Again, the stem keeps these two moieties in close proximity to each other, causing the fluorescence of the fluorophore to be quenched by energy transfer. When the probe encounters a target molecule, it forms a hybrid that is longer and more stable than the stem, and its rigidity and length preclude the simultaneous existence of the stem hybrid. Thus, the molecular beacon undergoes a spontaneous conformational reorganization that forces the stem apart and causes the fluorophore and the quencher to move away from each other, leading to the restoration of fluorescence. Unlike the TaqMan® probes, the probe will not be degraded. Such complex oligos are advantageous because of a high signal-to-noise ratio after hybridization, that is, efficient FRET quenching in the unhybridized state with decreased errant fluorescence, and a high specificity, that is, SNPs/single base mismatches can be detected. Molecular beacons do not require the exonuclease activity of a polymerase for fluorescent signal generation in qPCR and, under nonhydrolytic conditions, allow post-PCR melting curve analyses.

Scorpions® are a further refinement of the bifunctional probe principle (see Figure 3.6). They also contain a fluorophore and a quencher, but a primer is covalently linked to the probe at the 5′-end [58, 59]. The probe itself is designed as a Molecular beacon, a self-complementary stem sequence with a fluorophore at one end and a quencher at the other. However, the Scorpion® primer sequence is modified at the 5′ end. It contains a PCR blocker at the start of the hairpin loop. In the initial PCR cycles, the primer hybridizes to the target, and extension

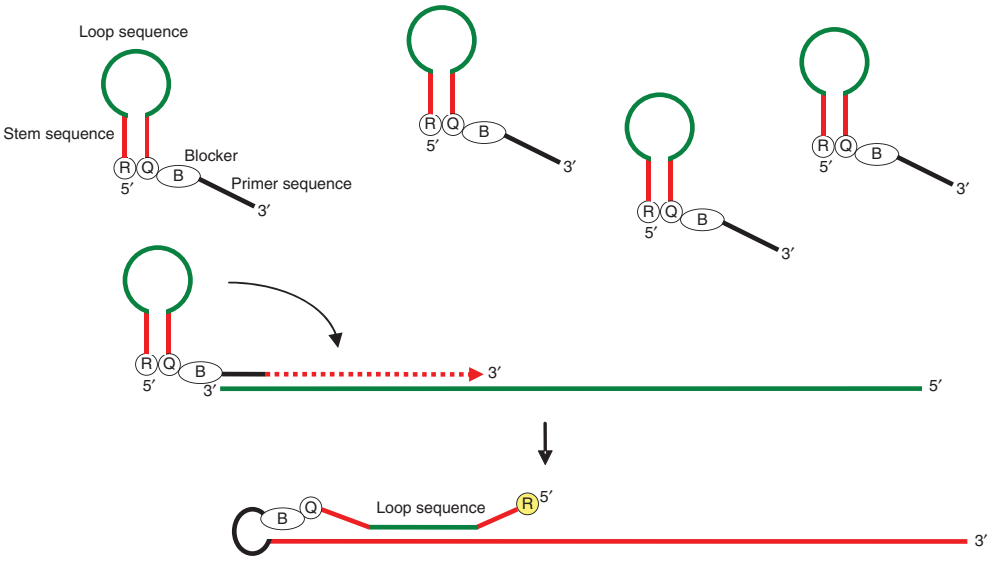

Figure 3.6 Modes of reporting III. Scorpions® combine priming and probing and also contain 5′ fluorophore (reporter; R) and central quencher (Q): the probe is designed as a molecular beacon but contains a PCR blocker at the start of the hairpin loop. After one cycle of PCR extension is completed, the newly synthesized target strand will be covalently bound to the same strand by the hybridizing loop (probe) sequence. This results in the separation of the fluorophore from the quencher.

occurs because of the action of the polymerase. After one cycle of PCR extension is complete, the newly synthesized target region will be covalently bound to the same strand as the probe. The melting of the hairpin loop requires less energy than the new DNA duplex produced. Consequently, the hairpin sequence hybridizes to a part of the newly produced PCR product. This results in the separation of the fluorophore from the quencher and causes emission. Because of the advantages of molecular beacons, Scorpions® are best suited for SNP analysis, qPCR, allelic discrimination, and genotyping. Notably, the best results are achieved when the primer pair is designed to gain an amplicon of approximately 100–200 bp; the stem T_m should be 5–10 °C higher than the probe T_m.

3.6.1 Quantification in qPCR

Quantification of amplicon synthesis in qPCR protocols can be achieved by following methods:

- Relative quantification is based on internal reference genes to determine fold differences in expression of the target gene.
- Absolute quantification gives the exact number of target DNA molecules by comparison with the DNA standards.

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DNA quantification by real-time PCR relies on plotting fluorescence against the number of cycles on a logarithmic scale. A threshold for detection of DNA-based fluorescence is set slightly above background. The number of cycles at which the fluorescence exceeds the threshold is called the *cycle threshold*, *Ct*. During the exponential amplification phase, the sequence of the DNA target doubles every cycle (n). The Ct value therefore corresponds to an amplicon number of 2^n. However, the efficiency of amplification is often variable among primers and templates. Therefore, the efficiency of a primer–template combination is assessed in titration experiments with serial dilutions of DNA templates to create a standard curve of the change in Ct with each dilution. The slope of the linear regression is used to determine the efficiency of amplification, which is 100% if a dilution of 1 : 2 results in a Ct difference of 1.

3.6.2
Melting Curve Analysis (MCA)

The melting behavior of dsDNA, that is, the dissociation into two antiparallel ssDNA strands, mainly depends on sequence, length, GC content, pH, and salt concentration. Labeled with a dsDNA-intercalating fluorescent dye (e.g., SYBR® Green or EvaGreen®) in qPCR analytics, the fluorophore signal decreases after the completion of the amplification cycles with increasing temperature because of the breaking of the base–base hydrogen bonds. The change of shape of the fluorescence/absorbance developing is monitored, and typically plotted as first derivative of the melting curve, $-\mathrm{d}F/\mathrm{d}T$ against T, in which T is temperature and F the fluorescence. A peak value is achieved at 50% strand separation (T_m) of a given sequence. MCA is part of the species-specific probe-based (FRET) result readout of the SeptiFast® sepsis pathogen detection assay (LightCycler®-based, Roche, Mannheim, Germany; see the following text).

Post-PCR melting curve analysis has become an important tool in qPCR diagnostics, but it is rather time consuming and prone to errors, and frequently leads to nonevaluable results. However, the state-of-the-art high-resolution melting (HRM) technique is sensitive enough to detect even SNP/point mutations and methylation profile aberrations. The most important HRM application, therefore, is gene scanning – the search for the presence of unknown variations in PCR amplicons prior to or as an alternative to sequencing (see also below under Section 3.7.1).

3.7
Controls, Probing, and General Aspects of Result Interpretation

While aqueous solutions are rather easy to handle, complex samples necessitate a more or less comprehensive preanalytical approach to make the analyte (i.e., target nucleic acids) available for detection and for the depletion of compounds that interfere with the results. For example, the state of the art in BSI pathogen

detection is nucleic acid trace detection directly in whole blood without blood preculturing to achieve higher pathogen cell/genome copy numbers. Cell lysis and further lysate preparation, that is, DNA isolation, desalting, and/or further (affinity) chromatography steps, demand individual process controls or an indicator of overall process validity, that is, an internal control. In general, the validity of an extraction, amplification, and detection of nucleic acids has to be ensured by definite controls [60], and inhibition must be detectable for each sample. If technically feasible and reasonable from a diagnostic point of view, each sample should be supplied with an internal control which also covers nucleic acid extraction (including cell lysis). In case of a qualitative proof of pathogens without rigid cell-wall structures, exceptionally a quantitative determination of the housekeeping genes may serve as control targets if validated accordingly [60], given that, even in case of a malfunction of the lysis step, the bacterial or fungal DNA will be isolated with sufficient yield. Proof of human targets, if available in the sample material (e.g., β-globin), is useful as semiquantitative inhibition control for PCR from blood, material from biopsies/swabs, and so on, and is already realized in some commercial NAT-based assays (e.g., Amplicor HPV PCR, Hoffmann-La Roche Ltd). However, human control DNA may vary in concentration, and a mere qualitative proof will give no information on partial inhibition. Better suited are target heterologs and homologs amplified in parallel or in the same vial (e.g., commercially available assays for *Chlamydia* spp., *Gonococcus* spp., human immunodeficiency virus (HIV), hepatitis B/C virus (HBV/HCV), PCR assays) [60].

Bacillus subtilis endospores, for example, have been tested as rigid genomic internal control DNA containers [61]. Modern, fully automated cartridge-based rapid ("bed-side") IVD (*in vitro* diagnostics) assays (e.g., Xpert® MRSA (methicillin-resistant *Staphylococcus aureus*) assay, Cepheid, Maurens-Scopont, France) also use internal control systems, and manual processing errors are largely prevented. In assay production, sensitivity of the whole assay protocol is usually tested on a regular basis via spiking experiments with total (viable) target cells.

The efficiency of DNA/RNA extraction and target amplification should be ensured with positive controls/internal inhibition controls in international units (e.g., colony forming units) in reproducible low copy numbers. It must be noted that internal controls may decrease the overall sensitivity of an assay by resource competition, and its influence on the test result therefore has to be validated in an optimal target concentration range.

Carryover of contaminants (whole cells, free nucleic acids including amplicons) have to be detected by negative controls without target sequences (preferably bacterial and fungal DNA-free), for example, water, buffer, or target-free clinical samples as negative control material. The latter is beneficial because of possible (human) carrier DNA fractions which allow better traceability of low DNA contaminations. Entrainment of pure reagent total process negative controls is often too costly and time consuming. However, an additional PCR-only negative control with reagents and primers but no target DNA makes sense in any respect.

Pure amplicon depiction by, for example, (agarose) gel electrophoresis and SYBR-Green-based qPCR is not accepted for diagnostic purposes. According

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to the recommendations of the German Microbiologically Infectious-Disease Expert Panel Quality Standards [60] as well as the claims of ISO 15189:2007-08 for medical laboratories (particular requirements for quality and competence specify the quality management system requirements particular to medical laboratories) and ISO/IEC 17025 (general requirements for the competence of testing and calibration laboratories), amplicons have to be analyzed and characterized by suitable molecular or biophysical methods with regard to their sequence to be used as diagnostic clinical evidence (see also [62]). In this respect, real-time PCR with subsequent hybridization by fluorophore-labeled probes in line with melting curve analysis, microarrays, DNA sequencing, or mass spectrometric analysis is best suited for NAT result confirmation. Two-step target amplification, for example, via nested PCR with amplicon-specific internal primers, or (enzymatic) amplicon restriction analysis is also appropriate [60]. Gel-electrophoretic amplicon depiction, traditionally combined with standard PCR, nowadays occasionally substituted by capillary gel electrophoresis, is not sufficient for amplicon delineation even under utilization of distinct length markers. Because of the fact that even amplicons with an overall high degree of conservation often possess domains significantly less conserved, mismatches with length markers designed after type strain target sequences are rather frequent and equal electrophoretic behavior is not reliably achieved accordantly.

3.7.1 Strategies in Amplicon Verification

3.7.1.1 (DNA) Microarray

In a (DNA) microarray, DNA probes are covalently spotted to a solid surface, for example, glass slides, silicon chips, or beads. In principle, hybridization between a labeled ssDNA strand of a melted PCR amplicon and a specific probe is visualized by a subsequent physicochemical reaction (color, precipitate). The signal depends on the stringency of the hybridization, as determined by the number of paired bases, the hybridization conditions such as temperature and salinity, and the amount of target bound.

Besides sequencing, the technique is a well-established procedure for amplicon verification, which can address, for example, several infective agents in parallel with low workload if automated. Systems are supplied by, among others, Affymetrix (Santa Clara, CA, USA) and Illumina (San Diego, CA, USA). Anagnostics (Anagnostics Bioanalysis GmbH, St. Valentin, Austria) has launched a fully automated microarray format, with the DNA (or protein) microarray spotted on cylinders instead of flat slides.

However, the microarray platform in general is technically hardly suitable for quantification because of a rather low dynamic range with often variable hybridization efficiencies and frequent false-positive/-negative results. The inherent drawbacks hamper widespread diagnostic application.

3.7.1.2 Flow Cytometry

The Luminex state-of-the-art approach in flow cytometry offers an alternative for multiplex PCR assay readout (Oosterhout NB, The Netherlands). This laser-based technology has originally been developed for cell counting, sorting, biomarker detection, and protein engineering. Analytes (RNA/DNA templates amongst others) are labeled with fluorescent ligands, suspended in a liquid, and passed through an electronic measurement unit for complex counting. Flow cytometry is routinely used in the diagnosis of health disorders, especially blood cancers, but has many other applications in basic research and clinical practice. Especially the xMAP® technology allows a high plex-grade for NAT-based target detection in microtitration plate scale [63]. Here, nucleic acids are extracted from target cells/several sample media and purified from the pretreated samples with commercially available kits. The target DNA is amplified in a single multiplex qPCR reaction, and the product is subsequently cleaned up to remove excess nucleotides and primers by an exonuclease I/shrimp alkaline phosphatase (Exo/SAP) protocol. In a third step, the purified amplicon mix is then subjected to primer extension which is specific for the infectious agent or the allele to be analyzed (ASPE or *target-specific primer extension*, TSPE). The amplified PCR fragment is only extended by a polymerase, which in addition incorporates biotin-labeled dNTPs if the 3′ end of the specific primer is bound to the homologous sequence. The 5′ end of the ASPE or TSPE primers is covalently linked to a universal tag sequence, which is then hybridized to the complementary anti-tag sequence coupled to a particular colored microsphere out of 100 combinable different-colored entities. The biotin unit is bound by a streptavidin/phycoerythrin conjugate. Inside the flow cytometer (Luminex® analyzer), lasers excite the internal red/infrared dyes of the microsphere and the phycoerythrin unit (reporter that marks the binding of the analyte). Both signals together allocate a specific PCR amplicon. The procedure requires some hands-on time and additional preanalytic effort. Experiences in direct pathogen detection are rather sparse and the overall sensitivity seems to be insufficient for direct trace detection of low-copy microbial DNA/cell counts [64]. However, the approach is quite smart for multiplexing applications and may be applied more routinely in the future.

3.7.1.3 Mass Spectrometry

Strain typing via PCR amplicon detection by mass spectrometry sheds new light on the application of broad range primers as a rather new development [65]. The Plex ID™ microbial screening workflow as implemented by Abbott (Plex ID™ Analyzer, Ibis Bioscience, Abbott Laboratories, IL, USA) (e.g., [66]), for example, starts with loading a nucleic acid extract (extraction to be done via in-house methods) onto a 96-well plate. Typically up to eight wells (multilocus singleplex PCR: one broad range primer pair per well) are used per sample, with the actual number of wells/primers used depending upon the breadth of coverage required. Primers target conserved regions that flank variable regions of highly conserved genes. After standard PCR amplification, the plate is then subjected to the mass spectrometry unit. Each amplicon solution is run through an automated clean-up

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process and injected into the mass spectrometer portion of the instrument. The amplicon weight for each sample well/primer pair used is determined, that is, the base composition is calculated from the mass of each amplicon present by a software algorithm, by electrospray ionization time-of-flight mass spectrometry (ESI-ToF MS) and compared with library entries to identify the organisms present. If unknown organisms with novel base compositions are detected, they are reported with reference to the closest known species in the database. Sequences for which no data bank entries are available will give no proximate information for the attending physician (which is true for all open systems; see also broad-range PCR with terminal amplicon sequencing) but will, over time, substantiate the data collection.

The approach provides the ability to distinguish between closely related species as well as giving information on SNPs or mutations that occur in the regions analyzed. If used only for pathogen identification, it would overcome the above-mentioned drawbacks of specific single-locus targeting without sequence information. Even downstream probing of amplicons will not provide information on close strain relations. Because of the constantly growing data bank, isolates of local interest can be identified with certainty. The contamination difficulty, as outlined above, of course remains. However, the sensitivity of these procedures, when compared to that of systems such as matrix-assisted laser desorption-ionization time-of-flight (MALDI-ToF) [67], is up to now rather low, even after sample preculturing/positive blood cultures as samples [68] (see the following text). Notably, procedures for sequence verification are in general only suited to increase assay specificity, only in exceptional cases sensitivity.

3.7.2 PCR Inhibitors

The influence of PCR inhibiting components has been comprehensively reviewed by Rådström and coworkers [69]. Inhibitors originate either from the original sample, or from sample preparation prior to PCR, or from both. Wilson [70] presented a systematic list of PCR inhibitors and stated that the mechanisms by which the inhibitors act inactivate the thermostable DNA polymerase, degrade or capture nucleic acids, or interfere with the initial cell lysis step. Important inhibitors belong to bile salts and complex polysaccharides in feces, collagen in food, humic compounds in soil, collagen, melanin, and myoglobin in tissue, polysaccharides in plants, proteinases and calcium ions in milk, and urea in urine. Beside heparin, hemoglobin in erythrocytes, lactoferrin in leukocytes, and immunoglobulin G (IgG) in plasma were identified as major PCR inhibitors in human blood samples [71, 72]. Inhibiting actions of those ingredients significantly depend on the handling of the samples. For example, because of the inhibiting actions of IgG on ssDNA, it is not advisable to use boiling as a sample preparation method or to use hot-start PCR protocols in case of blood specimens. Both hemoglobin and lactoferrin contain iron ions, which may be released into the PCR mixture and interfere with DNA synthesis. Heme, bilirubin, and hemin

as derivatives/cofactors of hemoglobin were also found to be NAT-inhibiting. Hemin was observed to be a competitive inhibitor with the target DNA and a noncompetitive inhibitor with the nucleotides through direct action against the DNA polymerase [73].

Takeover of inhibitors into the NAT reaction cannot be ruled out entirely even by an elaborate preanalytic course. However, DNA polymerases differ among their sensitivity to interfering compounds and may be chosen carefully. Higher DNA polymerase and/or lower DNA template concentrations, as well as additives, may also overcome the risk of inhibition. However, the most important inhibitor of PCR activity is an excess of (mostly human) background DNA within the reaction unit (see the following text).

3.8 Preanalytics

Avoidance of sample preculturing (e.g., in sepsis diagnostics, which at best affords that the total assay is as sensitive as the blood culture, albeit faster) and testing of complex samples necessitate a more or less comprehensive preanalytic sample preparation course. In other words, the best reasoned NAT protocol is worth nothing without a corresponding sample preparation. The main steps are, in principle, total cell lysis, for example, of blood cells and microbial cells in case of proof of microbial DNA in whole blood, and total DNA isolation, but might be expanded by directed human DNA degradation, microbial DNA enrichment, or chromatographic methods to scale down inhibiting fractions within the final DNA solution delivered to downstream NAT.

3.8.1 Sample Volume

The most expedient sample volume for NAT applications for BSI applications, for example, is still under discussion. While most assays for other sample materials than whole blood are designed for volumes below 1 ml by retained high assay sensitivities. Significantly higher volumes are subjected to sepsis pathogen detection: for example, SeptiFast® uses 2×1.5 ml, VYOO® (Analytik Jena, Jena, Germany) 5 ml of blood, and MolYsis (Molzym, Bremen, Germany) (only preanalytics) $\leq 1-10$ ml. The importance of the blood volume cultured or processed has been discussed long ago [74], but the implications of reduced sample volumes have been commonly accepted for the qPCR evaluation because of the always aspired miniaturization of assay layouts. Most liquid handling platforms on the market or even lysis devices address sample volumes ≤ 1 ml and, with regard to future standardizations of assay layouts, it has to be expected that the volumes will be in this range. However, sepsis pathogen diagnostics may be discussed gently as an exception. While, in general, it makes no sense to gain elevated sensitivity by increasing the sample volume in favor of turning alternative screws (e.g., optimized nucleic acid extraction, PCR, amplicon verification), the high volumes mark the awkwardness

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to cope with the extraordinarily low target concentrations in sepsis on one hand and the deficient signal-to-noise ratio on the other.

3.8.2 Cell Lysis

Release of DNA or RNA from cells implies the exposure to degrading nucleases that may be effectively inactivated by high concentrations of chaotropic salts dissolved in buffered aqueous solution to stabilize the nucleic acids within the lysate. Inhibiting proteins are also inactivated. Tissue samples have to be effectively homogenized in a convenient buffer medium.

An optimized procedure for microbial cell lysis within the sample is a crucial step in nucleic acid detection. To date, it lacks any standardization and produces markedly differing yields of microbial (bacterial plus fungal) DNA, and thus can significantly affect the results of the NAT methods chosen [75, 76]. Anyway, mechanical impact has been regarded as the most efficient lysis method for fungal cells [77, 78], whereas bacterial cells were in the past often disrupted using chaotropic buffers or enzymes/enzyme mixtures containing lysozyme, mutanolysin, and/or lysostaphin (e.g., for the lysis of *Streptococcus* spp.) [79]. Enzymatic/thermal lysis protocols were described as superior to mechanical impact [80], but such experiences should be mainly attributed to the mechanical devices used and their effective power, lysis matrix, sample-to-head space volume ratio within the lysis tubes, and the protocol (device settings) applied. It has to be considered that the last cited studies on cell lysis were done with rigid (fungal) cells devoid of human cell material, which markedly affects the lysis of the target cells, but the results of Fredricks and coworkers [76], which likewise support mechanical lysis, were obtained from spiked clinical samples, a technique that should basically be recommended for assay development.

However, the mechanical devices of the last generation (e.g., FastPrep®-24, MP Biomedicals, Eschwege, Germany; MagnaLyser®, Roche, Mannheim, Germany; Speedmill, Analytic Jena, Jena, Germany), which disrupt cells in several seconds to a few minutes while, for example, executing "figure 8" vertical angular motions (FastPrep®-24), enable standardized disruptions of designated rigid pathogen cells within clinical samples. The cells are ground between glass or ceramic bead – sand mixtures of particle sizes between 0.1 and 2.5 mm in diameter. Automated solutions combine cell lysis by shearing or enzymatically and downstream microbial DNA processing [81].

A smartly designed approach is based on a selective first-step lysis of human blood cells by chaotropic buffers and quantitative degradation of human nucleic acids via chaotroph-resistant DNases (MolYsis, Molzym, Bremen, Germany; [82, 83]). The enzyme is subsequently heat-inactivated, and bacterial pathogen cells are enzymatically lyzed in a second step to gain their genomic DNA for NAT detection purposes. It has to be questioned whether this procedure is actually a drawback of the analytical approach, because sub-lethally affected pathogen cells, due to prior antibiotic treatment and/or impact of the immune system, and

Gram-negative thin-walled cells may be disrupted within the first lysis step, which could lead to a total or partial loss of their genomic (target) DNA [84]. However, the kit passes direct comparison tests with competitive approaches, even though the tool may preferably be suited only for rigid cell types [85–87]. Notably, the preanalytic course of the multiplex sepsis pathogen detection kit Magicplex™ Sepsis Real-time Test (Seegene Germany, Eschborn, Germnany; details see the following text) for microbial cell lysis and DNA isolation is based on MolYsis. The automated magnetic bead-based protocol is performed with Seegene's SeePrep12 instrument based on NorDiag's Arrow lab automat (NorDiag ASA, Oslo, Norway). This system is known by the name SelectNA™ for automated MolYsis application by Molzym. The machine is a contained instrument for the extraction and isolation of highly pure DNA from pathogenic bacteria from blood and other body fluids (cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL), peritoneal, synovial, and other aspirates). The sample volume processed is 1 ml.

3.8.3 Nucleic Acid Isolation and Preparation

Traditionally, nucleic acid extraction methods have been time consuming and error-prone because of long hands-on times and improper handling, which yield products of poor quality and content. Contamination risks were high, and standard methods dealt with hazardous reagents (e.g., phenol and chloroform). Isolation of RNA was even more laborious than of DNA and only achievable with well-trained personnel. The major problem with RNA extraction is the ubiquitous nature of RNases, enzymes that rapidly degrade RNA. RNases may be difficult to eliminate, although with care and appropriate countermeasures reproducible extraction of high-quality RNA from important biological samples should be attainable [88, 89].

In general, the precision of the workflows were rather low. The ongoing trend for long accordingly is the coverage of a reduced number of handling steps by hazard-free, ready-to-use commercial kits, and a subsequent automation of the total course of action. Drawbacks of first-generation preanalytic devices were the potential for cross-contamination due to open plate sampling, aerosol formation, pipette leaks, and robotic failures. New-generation approaches keep sample dealings to a minimum, close, and individually, even in cartridges (see the following text), and warrant the increased reliability and repetitiveness demanded.

Nowadays, DNA and RNA are mostly isolated by adopting their binding to silica (glass), that is, silica gel membranes or suspensions, often spin-column-bound, silica-covered magnetic particles, which are preferably used in automated workflows, mostly in the presence of high concentrations of chaotropic salts (e.g., sodium chloride, perchlorate, guanidinium salts). The salts destabilize the hydrate shell of the DNA and support bonds between silica groups and the phosphate backbone. The usual steps are lysis, capture, washing, and elution. The last step is done with pure aqueous solution with high pH, which is beneficial in avoiding salt intake into the NAT reaction. Alternatives to silica are modes of anion

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exchange chromatography in which negative phosphate moieties of nucleic acids effectively bind to a positively charged matrix. This method is recommended for DNA > 100 kb. However, elution of highly purified DNA is done with high-salt buffers and subsequent precipitation prior to PCR.

However, it should be noted that the recovery rate of isolated nucleic acids depends on the DNA/RNA concentration within the (lyzed) sample. In samples with very low DNA content, it is recommended to add carrier-DNA to increase the rate of yield. A practical application could be, for example, the isolation of microbial DNA from whole blood with a standard precipitation-based protocol. In neutropenics with a leukocyte count below 1500 cells/µl, problems arise with human DNA concentrations $<5\,\mu g\,ml^{-1}$ if sepsis pathogen detection is aspired with up to $\sim 50\,fg\,ml^{-1}$ of microbial DNA in case of severe sepsis/septic shock (which approximates ~10 cfu/ml; see the following text).

The multiplex PCR-based assay (VYOO®, Analytik Jena, Jena, Germany) combines bead-based mechanical cell lysis with an extended multiplex PCR detection step [84]. Bacterial and fungal DNA is specifically concentrated by affinity chromatography while human background DNA is concertedly removed. Discrimination of eukaryotic and microbial nucleic acids by the host is achieved by immunocompetent cells via species-specific CpG motif recognition by the DNA-binding protein factor TLR9. This process is not restricted to this case but is also described for CXXC Finger Protein 1 or hCGBP [90, 91], a mammalian transcriptional activator that specifically binds unmethylated CpG dinucleotide motifs by recognition of the sequence [A/C]CG[A/C] resulting, from a statistical point of view, in an even higher number of potential binding sites [92, 93]. A matrix-immobilized truncated clone derived from CXXC Finger Protein 1 is used for affinity chromatography to relatively enrich the bacterial and, to a lesser part, fungal DNA content in blood samples from septic patients [94]. Downscaling of human DNA load and simultaneous elimination of PCR inhibitors promise to increase the sensitivity of chosen downstream detection methods by at least one order of magnitude [84].

3.9 Fields of PCR Application

3.9.1 Sequencing

An important impact is made in the field of nucleic acid sequencing methodology. The provision of sufficient amplicon material for recombination purposes (e.g., insertion of sequences into vectors), subsequent colony screening, and genetic fingerprinting, for example, for forensic personalization, paternity testing, or typing of organisms and uncovering their evolutionary relationships with high discriminative power, are performed routinely.

3.9.1.1 Pyrosequencing

Thus far, most DNA sequencing has been performed using the chain termination protocols developed by Frederick Sanger. This technique uses sequence-specific termination of a DNA synthesis reaction by modified nucleotide substrates. However, new technologies such as Pyrosequencing (Roche Diagnostics, formerly 454 Life Sciences, Branford, CT, USA; see also Qiagen/Biotage, Biotage AB, Uppsala, Sweden), which has enabled rapid genome sequencing, are gaining an increasing share of the market. More genome data is currently gained by pyrosequencing than by the Sanger method. A DNA polymerase reaction is combined with chemiluminescent light emission by a luciferase [95]. The new DNA strand is synthesized one base pair at a time with the code strand immobilized, and solutions of A, C, G, and T nucleotides are sequentially added and removed from the reaction. Light is emitted only when the nucleotide solution complements the first unpaired base of the template and pyrophosphate (PP_i) is released as a substrate for the luciferase. The sequence of dNTP solutions that produces light signals allows for the determination of the sequence of the template:

1) The ssDNA template is hybridized to a sequencing primer and incubated with the enzymes DNA polymerase, ATP (adenosine triphosphate) sulfurylase, luciferase, and apyrase, and with the substrates adenosine 5′-phosphosulfate (APS) and luciferin.
2) The addition of one of the four dNTPs (deoxyribonucleotides) (dATPαS, which is not a substrate for a luciferase, is added instead of dATP) initiates the second step. DNA polymerase incorporates the correct complementary dNTP onto the template. This incorporation releases PP_i stoichiometrically.
3) ATP sulfurylase quantitatively converts PP_i to ATP in the presence of APS. This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin, which generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a camera and analyzed using a program.
4) Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction can restart with another nucleotide.

Because of the limitations in target sequence length (only up to 300–500 nucleotides), pyrosequencing is most commonly used for resequencing or sequencing of genomes for which the sequence of a close relative is already available. However, the BlackLight® Sepsis Kit (2B BlackBio, Madrid, Spain) is based on pyrosequencing and subsequent data bank search of sequence information. The kit assists in the rapid identification of bacteria from positive blood cultures by providing a standard PCR to amplify three regions of the 16S gene to provide reliable templates for sequencing.

Emulsion PCR [96, 97], commercialized by SOLiD sequencing (formerly developed by Agencourt, later Applied Biosystems, now Life Technologies), isolates individual DNA molecules along with primer-coated beads in aqueous droplets within an oil phase. PCR then coats each bead with clonal copies of the respective

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DNA molecule followed by immobilization for later sequencing. The method is also adopted in the 454 Life Sciences pyrosequencing method [98].

Bridge PCR, alternatively used as pyrosequencing protocol for *in vitro* clonal amplification, also proliferates fragments upon primers attached to a solid surface, used in the Illumina Genome Analyzer [99].

3.9.2 Typing and Epidemiology

Multilocus sequence typing (MLST) is a technique for typing of microbial species by PCR fragment amplification of multiple (usually 7–10) housekeeping gene loci. About ~450–500 bp of each target are amplified to be accurately sequenced on both strands [100, 101]. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type. A series of profiles can then be the identification marker for strain typing. The large number of candidate alleles at each of the loci provides a high discriminatory power even for epidemiological investigations, and has great potential for automation and standardization. MLST databases contain the reference allele sequences and sequence types for each organism with the respective epidemiological data. Websites like *http://www.mlst.net/* contain interrogation and analysis software that allow users to query their allele sequences and sequence types. MLST is widely used as a tool by researchers and public healthcare workers.

A major target for PCR-based strain typing currently is *S. aureus*, which is an important human pathogen causing, for example, skin and tissue infections, pneumonia, and sepsis. The emergence of strains resistant to methicillin (MRSA) and other antibacterial agents has become a major concern, especially in the hospital environment, because of the high mortality rates after infection. The discriminative power of pulsed-field gel electrophoresis (PFGE), traditionally used as a gold standard for strain typing of MRSA, is generally higher than that of most sequence-based methods. However, single-locus DNA sequencing of repeat regions of the coagulase gene (*coa*), highly polymorphic due to point mutations and deletions/duplications of repeats, and the *Staphylococcus* protein A gene (*spa*) serve for reliable and accurate typing of MRSA. *Spa*-typing is especially interesting for rapid typing of MRSA in the hospital setting because it offers a higher resolution than *coa*-typing [102]. The SpaServer (*http://spaserver.ridom.de/*), designed by Ridom Bioinformatics (Germany), is a freely accessible database to add and download *spa*-repeat sequences and types. Also, MLST serves as a convenient tool for *spa*-typing but is not suitable for routine surveillance of MRSA because of the high costs and needed access to a high-throughput DNA sequencing facility.

As an alternative to bacterial and fungal strain typing via sequencing of repetitive regions, bioMérieux (Marcy l'Etoile, France) offers the DiversiLab® system based on *rep-PCR* (*repetitive sequence-based polymerase chain reaction*), a DNA fingerprinting technology in which differently sized amplicons of repetitive sequences are separated, yielding DNA fingerprints with high discriminative

value. As several of those elements are conserved in diverse genera of bacteria, a single primer set may be employed to fingerprint the DNA of many different microorganisms. DNA separation is automated on microfluidic chips.

3.9.3 Pathogen Detection in Complex Clinical Samples

NAT allows rapid target and (quite restricted) resistance detection within several hours, even from complex samples, usually within 4–6 h. The basis for NAT-based diagnostic applications is the detection of infectious agents and the discrimination of nonpathogenic from pathogenic strains by virtue of specific genes and/or highly conserved sequences such as the eubacterial 16S or 23S rDNA/rDNA spacer regions, and the pan-fungal 8S or 18S rDNA/RNA. In theory, free bacterial and fungal DNA and DNA from phagocytosed and free intact and nonviable pathogens are detected.

However, the effectiveness of PCR techniques is significantly impaired by the heterogeneity of the sample material with respect to nucleic acids (i.e., eukaryotic (human) bulk DNA, competing and contaminating microbial DNA), salts, and other blood ingredients (see the details mentioned above). Typically challenging sample materials for direct pathogen detection without preculturing prior to sequence amplification are whole blood, synovial fluid, and ascites. Because of the increasing importance of sepsis, pathogen detection directly in blood and the NATs claim to warrant an unrivaled rapid performance, and numerous methods have been developed to improve the diagnosis of BSI in recent years. Application is discussed exemplarily for current efforts in pathogen detection within complex samples and with respect to the ongoing discussion on the usefulness of NAT-based methods for respective trials at all.

Life-threatening microbial infections and their outcomes, namely sepsis and consecutive organ failure, are frequent complications of hospitalized patients. Sepsis results from the host's response to bacterial and fungal (and protozoan) infections, whereas a malfunction of the immune system is responsible for the development of organ dysfunctions and, finally, multiorgan failure. In Germany, about 60 000 patients die from severe sepsis, which, therefore, is one of the most frequent causes of death in intensive care units [103]. The incidence of sepsis has especially risen as a result of the use of invasive devices, aging of the population, and the higher incidence of immunosuppressive conditions.

Early directed antibiotic therapy, irrespective of the type of organism, portal of entry, age, or the occurrence of shock, is mandatory for successful therapy. Epidemiological data confirm that inadequate therapy leads to twofold higher mortality rates [104]. An increase of mortality of up to 8% per hour is proven in cases of delayed directed antibiotic treatment, irrespective of the causative bacterial or fungal pathogen [105–107]. Inappropriate initial anti-infective therapy for septic shock occurs in about 20% of patients, as reported in a recent study [108], and was associated with a fivefold reduction in survival. Almost 30% of proven fungal infections were treated inappropriately. This calls for the denotation of the etiologic

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microbial agent and its resistance profile at the earliest possible time. Knowledge of these parameters guides diagnostic procedures and reduces mortality, ICU stay, and antibiotic overuse [109]. In consequence, there is an unmet medical need for molecular diagnostic tests with superior positive and negative predictive values. None of the typical set of biochemical parameters, among others procalcitonin (PCT) and C-reactive protein (CRP), monitored in case of inflammation, functions as single independent criterion for sepsis and permits access to a directed antimicrobial therapy of the disease [110]. The gold standard technique, namely blood culture, which is the routine method in clinical microbiology laboratories so far to demonstrate the presence of pathogens in patients suspected of systemic infections, has drawbacks with regard to frequently fastidious or noncultivable organisms to be detected [29]. Blood cultures contribute only in the presence of viable and metabolically active cells and in the absence of antibiotic pretreatment.

Results on microbial presence needed for initiation of an adequate anti-infective regime are only in a minor part needed in a time frame below ~2 h, which is currently not achievable at all. Patients with advanced community-acquired sepsis (about 30% of sepsis incidences) are in part already empirically treated with broad-spectrum antibiotics at emergency units [111]. In most cases, hospital-acquired (nosocomial) early stage sepsis is treated under constant surveillance by culture-based methods, and timely pressure is lower to anticipate a meaningful culture- or NAT-based result on the underlying infective agent. However, broad-spectrum antibiotics are administered simultaneously or prior to blood withdrawal without trusted microbiological findings, which, in consequence, both enhances the development and/or spread of multiresistant organisms and reduces the success rates of blood cultures that are drawn at a later stage [112–114]. It is unlikely that even extremely fast diagnostic assays will redraft this course of clinical action within the first 2–4 h in near future for patients suffering from, for example, catheter-associated sepsis, as we will see in the following text.

However, PCR could significantly impact pathogen detection in cases of nosocomial septic infections where patients at risk, for example, those with artificial ventilation, urinary or central-venous catheters, peripheral venous cannulation, immunosuppression, or surgery, are monitored broadly from the beginning of the period of risk, that is, where sepsis could be diagnosed at an early stage with a higher likelihood of recovery.

3.9.4 General Aspects of Result Interpretation

3.9.4.1 Sensitivity and Specificity

Sensitivity and Specificity of NAT-based assays are the most important criteria for their usefulness in DNA trace detection. Sensitivity mainly depends on the ratio of the target sequence content to background DNA, that is, preferentially human DNA in microbial trace detection. Most assays on the market display a poor signal-to-noise ratio: crucial in pathogen detection from complex samples is the low abundance of pathogen cells within the circuit. The precondition is an

analytical sensitivity of the diagnostics which enters this analytical range to compete with the cultural standard techniques in the lab.

In cases of severe sepsis/septic shock, ≤10 cfu/ml of whole blood is to be expected in adults [29, 115] and ≤100 cfu/ml in newborns [29, 116]. Jordan and Durso [117] found up to 300 cfu/ml whole blood of neonates. Because of the fact that sepsis especially causes high mortality rates in newborns, particularly in preterm, low-birth-weight infants, and higher bacterial loads – which does not call for such an extraordinary high assay sensitivity than in adults – many NAT applications, mostly real-time PCR- and broad range primer-based, are related to pathogen detection in neonatal whole blood [116, 118, 119, 120].

Notably, it has to be considered that the number of augmentable cells is lower than the total number of cells that cover the nonviables. In the determination of colony forming units, inadequate separation of cell aggregates may lead to significantly lower colony numbers compared to viable cell numbers, mostly while performing plating techniques. Numbers of genome copies might, in addition, be indefinitely higher than colony forming unit numbers because of cells lyzed by the action of the immune system, which is beneficial for analytic purposes: ≤10 cfu/ml whole blood of septic adults presumably correspond to genome copy numbers ≫10 genome copies/ml, disregarding the fact that even in case of single-copy target genes the target number will be ≥1 due to DNA polymerization activity prior to cell division. In the case of multicopy PCR targets, the target numbers might be significantly higher.

It has to be kept in mind that, in case of severe sepsis or septic shock, only reduced benefit for the patient's outcome will be achieved at the later stages; pathogen detection should be initiated as soon as possible at lower bacterial loads (ideally down to <1 cfu/ml whole blood). Notably, the sensitivity for the detection of intracellular pathogens has reasonably not yet been defined, and no resilient information is available for the mean of whole blood microbial background DNA load of healthy individuals.

However, even ~10 cfu/ml of whole blood marks a level of concentration that is far below the limit of detection of most classical end-point PCR assays – and unfortunately absolutely in range with typical contamination loads of cultures and/or PCR mixes. In consequence, diagnostic methods are often declared to be rapid but rely, in fact, on preculturing of human samples to increase target numbers. Contaminants are indeed cultured as well, but assay systems depending on preculturing mostly exhibit a lesser number of process steps susceptible for contamination.

Real-time PCR devices are mostly regarded to allow the highest analytical sensitivity of NAT-based assays. The analytical sensitivity (≥3 cfu/ml has been determined for the SeptiFast® assay, Roche; [121]) generally depends on device and target, but also falls far short with low signal-to-noise ratios if applied without sample preincubation or further preanalytic measures when genomic DNA trace detection is done directly from whole blood.

Those drawbacks could be conquered by using higher starting material volumes combined with effective background DNA reduction while maintaining the

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total DNA load per PCR reaction – which, in other words, means increasing the target number per total DNA load or per amplification reaction. Human bulk DNA, co-isolated with microbial pathogen nucleic acids from the addressed sample material, is the cause for a minute pathogen-to-human DNA ratio by a factor of $\sim 10^{-9}$ cfu/ml whole blood, significantly reduced overall analytical assay sensitivity, and increased cross-reactivity with primers [122]. Sample dilution, of course, would reduce not only bulk DNA intake into PCR reaction but also target concentration. Increasing the reaction volume of the PCR also does not make sense: the PCR reaction depends on exact thermal control, which is only feasible in small reaction chambers. Total DNA load is therefore restricted.

3.9.4.2 NAT versus Culture

In case of NAT application in BSI diagnostics, there is presently no solution for the general problem of how to interpret result constellations where the pathogen DNA is detected by PCR but the corresponding blood cultures remain negative. In order to distinguish between "false" and "true" positive PCR results, additional microbiological findings and levels of inflammatory parameters are usually considered as a combined virtual gold standard. It has to be considered that false-positive PCR results may potentially increase unnecessary utilization of antibiotics. Therefore, a PCR result has to be critically judged in the clinical and microbiological context of the patient. A single detection of DNA of a species that belongs to the group of frequent contaminants but otherwise well-known causative pathogens, e.g., residents of the common skin flora, alone without combination with additional parameters should not immediately prompt or change antibiotic therapy. This is just as the rationale in transferring blood culture results into a therapeutic measure: blood withdrawal in case of sepsis suspicion should be done from two different peripheral locations by fresh puncture (from a lying central venous catheter exclusively under suspicion of catheter infection), each of 20 ml, to be split equally between aerobic and anaerobic bottles, not less than 10 ml per bottle, as defined by MiQ 3a 2007 [123]. Fungal cultures (e.g., using BD BacTec™ Mycosis IC/F flasks, BD Diagnostics, Becton Dickinson GmbH, Heidelberg, Germany) have to be administered additionally.

Microbial species belonging to the residential skin flora (e.g., *Corynebacterium* spp., *Bacillus* spp., *Acinetobacter* spp., *Staphylococcus* spp., *S. aureus*, together with *Escherichia coli* due to frequent smear contamination by inadequate hygiene measures) are only valued as true positives if grown in the bottle sets from both drawing sites to reduce false negative counts. However, analysis of more than one NAT sample withdrawn from more than one site in search for sepsis pathogens is currently not common practice even in prospective studies on the suitability of new diagnostic approaches.

At present, the discussion on how a NAT-based finding has to be judged at all is still ongoing: a positive result in DNA targeting may not necessarily provide evidence either for the presence of viable cells or for the correctness of the proof of a disease-causing pathogen. Likewise, the determination of a more or less correct total pathogen cell number does not provide any information whether a pathogen

has the ability to cause an infection. On the other hand, the finding of designated pathogens (e.g., vancomycin-resistant enterococci, VRE) should excite an alert in any case.

Clinical studies determine the (clinical) relevance, assay sensitivity, and specificity for distinct targets, that is, of clinical pathogen strains – in comparison to the analytical validation in research labs with type strains. The number of septic patients infected by true positives and included into a respective study must be large enough to cover all targets with statistical relevance: even in tests with high sensitivity and specificity, the positive predictive value (PPV) might be low if the prevalence is low, given that the test sensitivity is the ability of a test to correctly identify true positives and that test specificity is the ability of the test to correctly identify true negatives. Conversely, despite high PPVs, negative predictive values are often not sufficient to securely rule out infection. In BSI diagnostics, for example, positive NAT findings have to be correlated with other clinical observations and diagnostic tests by clinicians, and cannot be the sole reason for any therapeutic consequences. In conclusion, clinical trials will actually hardly gain sufficient information on the suitability of chosen conserved regions for primers/probes of a single target as part of a panel designed for addressing infrequent but multiple targets. This is a problem of all NAT-based assay systems on the market using species-specific primers/probes for pathogen detection, first of all for multiplex assays but rather not for test systems depending on broad-range primers.

3.9.4.3 The Fate of Antibiotic Resistances Detection

Clinical and commercial success of NAT-based methods in clinical pathogen detection mainly depend on their ability to compete with standard (i.e., culture-based) methods in antibiotic resistance detection [124]. This becomes increasingly important in the context of rising broad-spectrum resistance in Gram-negative rods, particularly for carbapenemase-expressing pathogens such as *Klebsiella* spp. and multidrug-resistant *Acinetobacter baumannii* [125, 126]. These pathogens are resistant to almost all antibiotics except colistin and to a lesser degree tigecycline [125, 127], which are not part of the recommendations of current major guidelines for empiric treatment. Carbapenemases (e.g., NDM-1) encoding genes are plasmids located and easily spread over species barriers [128]. A Greek study recently showed that initial inappropriate treatment of those carbapenemase-resistant *Klebsiella pneumoniae* strains results in a twofold increase in mortality [126]. Fast identification of MRSA or VRE may be feasible by molecular methods because methicillin and vancomycin resistances are encoded by a limited number of known genotypes. However, many types of bacterial resistance based on several distinct point mutations, for example, penicillin resistance in *Pneumococcus* spp. and more than 300 extended-spectrum β-lactamases (ESBLs) described so far for distinct Gram-negatives, are encoded by a huge and still evolving variety of genotypes. Moreover, phenotypic resistances and the number of various regulatory genes, whose role in resistance expression remains elusive, preclude simple molecular- (or proteomic-) based economic

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detection approaches for routine purposes. Therefore, molecular detection of the most prominent bacterial antibiotic resistance-mediating target genes, one of the most relevant aspects for a timely confirmation or correction of the initial therapeutical regimen, will be restricted to a limited number of resistance types in the foreseeable future, and broad antibiograms remain subject only to the culture-based approach. This is also true for add-on information on antibiotic minimal inhibition concentrations (MIC) in the context of therapeutic drug monitoring (TDM), which is discussed as a key intervention to improve outcomes in patients with sepsis and septic shock and which becomes increasingly important. On the other hand, therapy-relevant NAT findings remain indisputable and most welcome if other therapeutic measures to save the patient's life are exhausted.

In consequence, with no solution to establish a meaningful resistogram without culturing, and the ongoing uncertainties in the valuation of NATs in pathogen detection, only combinations of diagnostic findings should be the basis of therapeutic measures, and molecular pathogen detection can only be an adjunct to cultural pathogen and resistance profiling. Considering that the blood culture's positivity rate is <10% in patients with severe sepsis/septic shock on German ICUs [103], this estimation is rather deflating.

3.10 Microbial Trace Detection in BSI, Ascites, and Synovial Fluids

Although most of the published PCR-based approaches have been useful for the identification of bacterial species at large, they identify only one species at a time, notwithstanding the fact many of them rely on a multiplex-compatible probe hybridization procedure that incurs time and expense. An economically more efficient approach is, besides the drawbacks mentioned above, the application of protocols capable of identifying broad panels of relevant species in a highly parallel fashion, which is especially demanded for identification of the underlying causative microbe of a sepsis episode out of a multitude of suspects. The most conversant diagnostic approach in sepsis pathogen detection is still the multiplex real-time PCR-based assay SeptiFast® (Roche Diagnostics, Mannheim, Germany), which covers 25 bacterial and fungal species (approximately 90% of nosocomial bacteremia causative species) by FRET probing combined with melting point analysis, directly from whole blood. Therapy-relevant information is gained in less than 4 h for species detections plus another 2 h for mecA resistance detection. Lehmann and coworkers [121] used the current assay layout in which the target DNA is amplified in three parallel reactions (according to Gram-positive, Gram-negative, and fungal primer pools) using Hot-Start Taq polymerase. Patented *Internal Transcribed Spacer* regions (*ITS*) between the 16S and the 23S ribosomal DNA sequences of Gram-negative and Gram-positive bacteria and between the 18S and 5.8S ribosomal sequences of fungi were used as targets. The sequences were amplified with either universal or specific primers. Results showed detection rates of 50–100% for selected clinically relevant bloodstream pathogens with analytical sensitivities between 3 and 30 cfu/ml

whole blood and an overall specificity of 98.8%, as compared to conventional clinical microbiology data.

This protocol, however, is comprehensible owing to the restricted multiplex ability of qPCR due to the limited availability of wavelength-specific fluorescence dyes and/or fluorescence channels of the device used. This is the reason for the rather short SeptiFast® target panel: the number of dyes is in fact growing with the state of the art, but to achieve a still limited but increasing multiplex applicability, fluorescence detection comes along with melting point analyses and is done with high hands-on workload [28]. However, launched in 2006, more than 60 scientific publications on the clinical application of SeptiFast® have been announced so far, more than on any other comparable system on the market. Despite the apparent limitations with regard to performance and data interpretation, for example, high probability of false-positive results, above all reported for *coa*-negative staphylococci which may lead to an adverse antibiotic regimen, failure to detect relevant pathogens, and overall costs, progress has been reported with respect to evaluation of general clinical utility [129] and a positive impact on therapeutic decision making by additional qPCR performance directly at the onset of a systemic inflammatory response syndrome [130]. Nevertheless, the latest publications on the use of the assay point toward a common trend of the general applications of the designated sepsis molecular diagnostics, which at the end is in conflict with their approach of a receptive (sepsis) market: more and more studies present clinical data after adoption of the assays beyond their original intended use.

The LightCycler® application has been used for the detection of microorganisms in purulent fluids from patients with pyogenic infections [131], in urine [132], ascetic fluids [133], and cardiac valve tissues [134]. Obviously, new fields of application are demanded not only for reason of business development but also due to the fact that PCR application is impacted by the above-mentioned side effects. The VYOO® assay, originally intended for sepsis pathogens in whole blood, has also been applied in pathogen detection in ascites in cases of spontaneous bacterial peritonitis (SBP, [135]) and synovial fluids [136], in which pathogen cell numbers are probably not as low as in blood of septic patients.

According to current pathogenetic concepts, SBP occurs as a result of bacterial translocation from the gastrointestinal lumen to the mesenteric lymph nodes, with subsequent systemic circulation and seeding into ascites. The detection of ascitic bacterial DNA using the LightCycler® SeptiFast® Kit MGrade was questioned to serve as a diagnostic tool for SBP and for episodes of bacterial translocation [133]. The authors reported the identification of ascitic bacterial DNA as an appropriate alternative to bacterial ascites culture for pathogen identification in patients at risk for SBP. However, in contradiction to Bruns and coworkers [135], Appenroth and coworkers found no evidence that detection of bacterial DNA in ascites of patients with liver cirrhosis is of clinical or diagnostic relevance, but patients with SBP showed clinical signs of infection.

Proof of bacterial DNA might be of higher relevance in the diagnosis of chronic prosthetic joint infection (PJI). Currently, no consensus exists on the definition of criteria required for microbiological identification while loosening of orthopedic

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implants, caused by infections and aseptic mechanical alterations, which implies important medical and socioeconomic problems. Periprosthetic biopsies (PPBs) suffer from inadequate sensitivity, and infections are mostly detected by quantification of neutrophile granulocytes. Molecular methods are demanded for the detection of pathogens (and resistances) responsible for PJI but their usefulness is under discussion with regard to the high numbers of false positives. However, it has become generally accepted that the diagnosis of infections in patients with arthritis and/or joint prostheses requires interdisciplinary cooperation and the use of up-to-date methods. Meanwhile, besides VYOO®, also the Roche assay has been used for pathogen detection in synovial fluids [137] and the authors confirmed the potential of the current diagnostic approach, especially for patients undergoing antibiotic treatment.

A further study focused on synovial fluids, PPBs from the joint capsule, the interface membrane, and specimens from the surface of the explanted prosthesis, and no antibiotics were allowed prior to sample drawal [138]. Interpretational criteria were applied to differentiate a true-positive PCR from potential bacterial DNA contamination. In the end, 16S rRNA gene real-time PCR did not identify more cases of septic prosthetic loosening than did culture of adequate PPBs.

In daily practice, instances of patients pretreated with antibiotics prior to sample withdrawal are frequent, which, in consequence, leads to increased numbers of (false) negative cultures. In this respect, NAT may gain cumulative practical value, which was not foreseeable several years earlier when the effectiveness of PCR in prospectively detecting infection in failed joint replacements was regarded to be worse [139]. Synovial fluids were intraoperatively collected from prosthetic joints and examined by broad-range PCR. Results were compared with the clinical diagnosis of infection based on the results of blood tests, preoperative joint aspiration, culture and histology of multiple intra-operative tissue samples, as well as on surgeon's assessment. However, the sensitivity of the technique was 92%, the specificity 74%, the accuracy 76%, the PPV 34%, and the negative predictive value was 98%. The authors concluded that this PCR technique could not be recommended for routine work because of the large number of false positives (presumably due to contamination) or bacterial presence related to low-virulence organisms, low bacterial load, or a strong host immune response.

As mentioned above, qPCR is regarded as the PCR technology with the highest sensitivity (lowest detection limit). While the Roche LightCycler® was state of the art for long time in clinical settings, other systems have entered the qPCR market successfully (e.g., Applied Biosystems® Real-Time PCR Instruments (Life Technologies), RotorGene® (Corbett Life Science/QIAGEN), SmartCycler® (Cepheid), QTower 2.0 (Analytik Jena), TOptical (Biometra)). However, presumably with regard to patent restrictions, only Seegene (Korea) has launched up to now a new real-time PCR-based sepsis pathogen assay (Magicplex™ Sepsis Real-time Test, see mentioned above) which covers more than 90 pathogens as well as the typical three drug resistance markers (*mecA*, *vanA*, and *vanB*) directly from whole blood. The huge but questionable panel of the Seegene sepsis kit is only achievable with a multilevel amplification protocol: typing of a respective

strain is done after a previous qPCR group screening, that is, using Gram (±) bacteria/fungi and drug resistance primer pools, with oligoplexed primer pools (e.g., selected for *Streptococcus* spp., *Staphylococcus* spp., or *Enterococcus* spp.). However, to enter the addressed range of sensitivity, the two-step quantitative amplification process is still not sufficient: the very first amplification step is done with a standard PCR device and two (oligoplex) pan-primer pools. Taking into consideration that further equipment for automated magnetic bead-based cell lysis and nucleic acid extraction is needed, it is obvious that sepsis diagnostic currently calls for high initial investment, complex device compilations, and considerable lab space for storage and handling.

To gain higher specificity, Seegene has launched their assay with a new primer design, called *dual priming oligonucleotides* (*DPO*™), which contain two separate priming regions joined by a polydeoxyinosine linker. The linker forms a bubble-like structure which itself is not involved in priming but rather delineates the boundary between the two parts of the primer. The structure results in two primer segments with distinct annealing properties: a longer 5′-segment that initiates stable priming, and a short 3′-segment that determines target-specific extension. DPO™-based systems block the extension of nonspecifically primed templates, and thereby generate increased PCR specificity even under less optimal PCR conditions [140]. In the study of Chun and coworkers, conventional primer systems reduced the occurrence of nonspecific products by increasing the annealing temperature, while the DPO™ primer produced only one target product over a wide range of temperatures. The product promises to allow for single base discrimination, specific and simultaneous analysis of multiple SNP sites (DPO™-SNP), and detection of multiple pathogens without any false results. However, resilient data are so far only available for the detection of viral nucleic acids [141] and for broad-range amplification of the bacterial 16S rRNA gene from human clinical specimens [33]. The latter article is quite promising with a view on a reduction of overlayed sequences after broad-range PCR (see mentioned above).

3.10.1 Grown Blood Culture Media as Samples

Besides the above-mentioned procedures, which cover the whole workflow from complex sample to final result readout, easy-to-handle, short, PCR-based procedures are offered for sampling of grown blood cultures. These approaches possess features of the blood culture with respect to detection of viable, nonfastidious cells only, but without the need for complex preanalytical operation. Those assays are often combined with a DNA microarray. Again, an all-out antibiotics susceptibility pattern is not provided.

Pathogen detection in positive cultures via multiplex PCR and downstream strip-based DNA microarray technique is offered by Mobidiag (Mobidiag Ltd, Helsinki, Finland; Prove-it™ Sepsis StripArray), which allows the identification of up to 80 bacterial and fungal pathogens (and mecA) by broad-range PCR followed by specific identification on a microarray [142].

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An alternative with respect to basic equipment needed and overall low assay costs is given with the standard multiplex PCR-based ELISA assay (Hyplex® BloodScreen Multiplex, Amplex BioSystems GmbH, Gießen, Germany), which admittedly covers only a small panel of clinically important pathogens at the species level and several resistance genes [143]: detected are the most common bacterial pathogens responsible for sepsis, but with alternatively pooled primers, for example, multiresistant staphylococci (MRSA), community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA), VRE, *Mycoplasma* spp., ESBL or carbapenemases producing bacteria, or important *Candida* species. Several different specific DNA regions are amplified for a single target and the amplicons are reversely hybridized in commonly used microtiter plates similar to a traditional antigen ELISA, warranting high specificity. The readout is easily automatable vie ELISA processors, which is a significant benefit for routine use.

3.11 Upcoming Routine Solutions – Dawn of Assay Automation

The trend for increasing automation of lab processes has also conquered the clinical NAT branch for some years. At last, some aspects, products, and future aims are touched briefly.

Current challenges in the transfer of innovative NAT-based assays in (clinical) routine mainly focus on automation of process flows. While several studies on the clinical impact of the above mentioned designs are available or ongoing, routine use is currently out of scope due to the lack of standardization and resilient predictive values, the ongoing discussion on the interpretation and rationale of the diagnosis DNAemia in general, low operational capacity, and in part not acceptable times-to-result and/or hands-on-times. Such assay-specific process times have not been given in this chapter because these time frames are constantly decreased by manufacturers in product upgrades. Most workflows that cover all steps between pre-analytics and final readout last several hours (3.5–8 h), depending on the techniques used, with differing hands-on-times within, lasting from several minutes in totally automated, cartridge-based offers (see the following text), up to hours in manual or semiautomated procedures. Those are difficult to apply in routine clinical use and call for skilled and hence expensive technicians. In the end, the hands-on-times deliver the inclusion criterion for the clinical routine acceptance.

The number of handling platforms suited for individual NAT protocols and their preanalytics is growing fast. Most platforms offer cooling/tempering items for samples, buffers/reagents, and products. High-priced devices provide standardized communication interfaces for bidirectional data exchange with laboratory information (management) systems, (LI(M)Ss), work lists, and rack files upload (e.g., QIAsymphony, QIAGEN, Hilden, Germany; TIGRIS® DTS™ System, Gen-Probe Incorporated, San Diego, CA, USA; Viper™ System, BD, Franklin Lakes, NJ, USA; Plex ID™ Analyzer, Ibis Bioscience, Abbott Laboratories, IL, USA). Bar code scanning of samples and reagents enables full sample tracking throughout the entire purification and assay setup procedures. Due to high risks of contamination,

the work stations are placed in hoods and are provided with tip guards preventing cross-contamination and built-in UV lamps. Chronological electronic documentation of results, including information about sample IDs, their positions, lot number, expiry date of sample preparation, and PCR reagents, is provided.

Most systems pile up masses of one-way disposables. After all, the use of sample-specific cartridges for pre-analytic and/or analytic sample treatment and complex consumables decreases hands-on time, pipetting steps, and contamination risks to a minimum. Such systems do not contain unsealed reagents or waste. Most platforms are featured with two or more (cartridge) slots, or devices can be stacked or placed side by side, which allows processing of cartridges completely independently (random access) and increasing system throughput capacity as needed. However, the assays/technologies are only in part FDA-cleared and/or qualified for IVD (*in vitro* diagnostic) use, but are suited for research use only (e.g., Plex ID, Abbott).

Cepheid's GeneXpert® System (Cepheid Europe, Maurens-Scopont, France) has been the first commercial system to fully automate and integrate all the steps required for PCR-based DNA testing. Test results are provided from a raw sample in about an hour [144, 145]. However, apart from its rather small target panel, there is no relevant multiplex application, especially for sepsis. The device integrates sample processing, nucleic acid amplification, and detection of the target sequences using real-time and RT-PCR and is equipped with a PC and barcode scanner. It employs single-use disposable cartridges that hold PCR reagents and hosts the PCR process.

New, fully automated, cartridge-based platforms are supplied by Curetis (Unyvero Solution, Curetis AG, Holzgerlingen, Germany) and Biocartis (Biocartis NV, Mechelen, Belgium). Curetis focuses on severe bacterial infections, providing a solution for pneumonia testing (Unyvero P50 Pneumonia Cartridge) with microarray-equipped cartridges suited for several sample types. After sample lysis, the user assembles the cartridge by simply inserting a tube with the lyzed sample and a second tube containing the (PCR) master mix – no further hands-on steps are necessary. Once assembled, the cartridge is a physically closed system – avoiding any possibility of spilling sample fluids or reagents and minimizing contamination risks. The disposable cartridge is compatible with standard waste disposal procedures of hospitals and laboratories.

While Unyvero is based on end-point (standard) PCR, Biocartis' Molecular Diagnostics Platform uses multiplexed real-time PCR amplification. The multitude of sample types that are compatible with the Biocartis cartridge demands specific pretreatment. For materials such as sputum or feces, the viscosity of the sample is quite high, while others (e.g., fresh or paraffin-embedded tissue) are completely solid. Before such sample types can be adequately processed and handled by fluid handling systems, they must be liquefied and homogenized for the microfluidic process flow. The core of the technology consists of silicon microparticles coated with specific capture molecules.

Besides platforms that offer automated protocols for both preanalysis and NAT/sample readout, liquid handling systems are available that are suited only

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for sample preparation in view of downstream PCR processing. Most devices are open systems for various workloads and sample types, and address a 96 micro-titration plate format. Examples are the VERSANT® kPCR Sample Prep bench-top platform (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) for nucleic acid extractions from samples such as plasma, serum, urine, dried blood, stool, and breast milk, with a universal reagent kit. As an example, the CLART system (GENOMICA, S.A.U., Madrid, Spain) is a low-density, microarray-based platform for clinical use that allows the detection of multiple targets in a single test. Sample processing and analysis/interpretation of results are performed automatically.

Liquid-handling robots for processing of part or higher volumes via easy-to-use protocols for nucleic acid (and protein) purification are supplied by Thermo Fisher Scientific Inc. (KingFisher® Flex), PerkinElmer Chemagen Technologie GmbH (chemagic Magnetic Separation Module I, Chemagic Star), Promega (Maxwell® 16), Invitrogen (iPrep™), Qiagen (QIAcube®), and AnalytikJena (C16 and C96). These robots are an easy first step toward automation for laboratories that have traditionally processed all of their samples manually. For larger numbers of samples and sample processing in 96- and 384-well plates, automated liquid handlers are supplied by Beckman Coulter (Biomek® 3000 Laboratory Automation Workstation), Eppendorf (epMotion®), Tecan (Freedom EVO® 75), Hamilton Robotics (MicroLab® NIMBUS), Caliper Life Sciences (Zephyr® Compact Liquid Handling Workstation), and AnalytikJena (FasTrans).

NorDiag recently launched a magnetic sample preparation protocol (Bugs'n Beads™ (BnB), NorDiag ASA, Oslo, Norway), which uses specially coated paramagnetic particles capable of isolating microorganisms from complex samples (including urine, cell culture, and feces) for downstream processing of nucleic acids, for example, for PCR/SDA/NASBA and other methods of detection. The cells specifically adhere to magnetic particles, which is followed by magnetic separation from the sample material and most of inhibiting compounds after a short incubation. The cells are sequentially disrupted and nucleic acids are released, followed by immobilization onto the same magnetic particles. This allows for the cell debris to be removed, leaving behind pure nucleic acids. Purification of bacterial DNA from *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, and *Ureaplasma urealyticum* can be carried out simultaneously.

3.12 Conclusion and Perspective

In recent years, a constantly growing number of NAT-based methods for the detection of infective pathogens have been launched. Even an overview on the most recent applications for fungal, viral, and bacterial detection would go beyond the scope of this chapter. Remarkable is the incremental relevance of, for example, non-PCR protocols, most notably in the field of viral infections. Improved complex sample pretreatment strategies, simplification of the analytical

course, and a further automation of process modules or complete protocols are areas of current development.

NAT has been an important basis of sequencing strategies for long. However, it may be displaced in the future by nanopore sequencing [146]. Nanopores were originally built of membrane-bound proteins (e.g., alpha hemolysin (αHL), *Mycobacterium smegmatis* porin A (MspA)), which shape a hole of about 1 nm internal diameter, but artificial pores have been made of silicon meanwhile [147]. However, when immersed in a conducting fluid and supplied with a potential difference, an electric current due to conduction of ions through the nanopores can be measured with high sensitivity. The current is sensitive to the size and shape of the passing molecules: if linear polymers (e.g., DNA) pass through, for example, forced by electrophoresis through a nanopore membrane, the conductivity changes depending on the succession of monomers [148]. As an example, Oxford Nanopore Technologies (Oxford Science Park, Oxford, UK) develops protein and solid-state nanopore technology with the aim of sequencing DNA and identifying biomarkers, drugs of abuse, and a range of other molecules. They launched their first working station in February 2012.

Although south Asian companies such as Seegene have conquered the European market with pretended new assay strategies in sepsis pathogen detection, actually no fully satisfactory solutions for routine application are on hand due to the intrinsic challenges that might be difficult to solve within the next years. NAT-based tools may be adjuncts in special problem areas (e.g., neonatal and fungal sepsis) or as last resource in saving patients' lives if alternative clinical approaches fail. A standardization, however, is not yet in sight. Up to now, only sparse information is available on potential cost benefits for routine application of molecular diagnosis versus conventional detection of bloodstream infections.

However, considering the enormous analytical potential of nucleic acid amplification technology and the dynamics in various ongoing research and development activities, it should just be a matter of time before new molecular methods capture their predicted prominent role in routine BSI pathogen diagnostics. Future technological improvements may lead to a reverted situation, where molecular methods have evolved as the gold standard and their limitations are supplemented by the results of culture methods. The cornerstone for a broad and promising clinical use of PCR-based methods has been placed several years ago. The advent of successful sepsis diagnostics has begun.

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4
DNA Microarrays for Pathogen Detection

Holger Schulze, Maya Rubtsova, and Till T. Bachmann

4.1
Introduction

Since its invention at the end of the last century [1, 2], the DNA microarray technology has created a huge interest among people interested in infectious diseases, especially for diagnostics. The technology itself was repeatedly and extensively described in many textbooks and reviews and will not further be described here. For a thorough coverage of the technology, its applications, and the bioinformatic aspect of data analysis, see, for example, the three Chipping Forecast Supplements to *Nature Genetics* (Chipping Forecast Editorial, 1999; Chipping Forecast II Editorial, 2002; Chipping Forecast III Editorial, 2005). Basically, DNA microarrays are used to detect nucleic acid targets using nucleic acid probes which are bound in an array format to solid supports, making them easy to follow. The binding of nucleic acid targets, which are usually amplified by nucleic acid amplification strategies such as PCR (polymerase chain reaction), to these probes is conventionally followed by fluorescence signals and used to identify the organism they are originating from. For infectious diseases, these organisms are usually pathogens and therefore of greatest importance to healthcare. The application of DNA microarrays for pathogen detection has become a widespread field of research which was repeatedly reviewed, and a number of commercial products have been developed and launched. For some reviews on this topic, see [3–9]. The main driver for molecular diagnostics in infectious diseases is that culture-based testing, although still being the gold standard, is too slow because it can take 2–15 days until a result is received. In contrast to methods based on culture, molecular methods, and especially DNA microarrays, enable fast identification of the etiologic agent in infectious diseases. In addition, these methods allow the user to detect additional information relevant to the treatment decision which may be related to antibiotic resistance or virulence factors. DNA microarray technology appears to be an ideal choice for this purpose, as it enables a highly parallelized testing to a great information depth.

Although the traditional disciplines in infectious diseases following pathogen classes become increasingly less obvious, we decided to review the available

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literature separated in diagnostic microarrays for bacteria, viruses, fungi, and parasites because the body of DNA microarray literature on infectious disease diagnostics is very large (e.g., a narrow search in PubMed for "DNA microarray infecti* diagnostic" reveals 1490 publications (October 2013)). For the same reason, we mostly limited the search to the last 5 years. These publications are discussed in the sections which are accompanied by tables summarizing the key features of the described systems. A main aspect of this review is the consideration of commercially available DNA microarray-based diagnostics. We discuss these products in the sections for the respective pathogen class but summarize the commercial systems at the end of this overview in a table organized by company offering the product.

4.2 DNA Microarrays for the Detection of Bacterial Pathogens

In recent years, a large number of DNA microarray tests for bacterial species identification have been developed for several clinical applications such as sepsis, pneumonia, and infectious diarrhea diagnostics or for the identification of certain pathogens such as *Mycobacterium* species [10], *Escherichia coli* [11–14], chlamydiae [15], and several other bacteria [16–21], as shown in Table 4.1. In the following Section 4.2.1 some examples of clinically validated microarray tests will be described in more detail.

4.2.1 Major Bacterial Diseases

4.2.1.1 Blood Stream Infections/Sepsis

Sepsis and pneumonia in adults and children are life-threatening diseases with high rates of morbidity and mortality worldwide. Of 7.6 million children who died in the first 5 years of their life in 2010, 14% died of pneumonia and 5% died of sepsis or meningitis [37]. Consequently, blood stream infections are a major target researchers aimed to address using DNA microarray-based pathogen detection. Wiesinger-Mayr *et al.* developed a DNA microarray test for the detection of 25 bacteria and fungi relevant for blood stream infection. The test for sepsis-causing organisms combined multiplex PCR with universal 16S rDNA and 18S rDNA specific primer pairs with hybridization of the fluorescence-labeled PCR products on a microarray consisting of 76 species-specific DNA probes. The time to result from pathogen isolation from blood samples to species identification was 6 h. The low sensitivity toward some bacteria, such as, for example, *Staphylococcus aureus* (limit of detection (LOD) = 10^5 cfu/ml), represents a barrier for clinical application. In contrast, the LOD for *E. coli* detection in blood samples was 10 cfu/ml. Palka-Santini *et al.* developed a DNA microarray combined with a large-scale multiplex PCR with primer pairs to amplify up to 800 different gene segments from nine pathogenic species. The PCR amplicons corresponded to

Table 4.1 DNA microarrays for bacterial species identification.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[11]	JP	*S. aureus*, *E. coli*, *S. enterica*, *P. aeruginosa*	Reference strains	None	On-chip PCR directly from bacteria without DNA extraction, 23S rDNA amplification followed by on-chip primer extension, enzymatic precipitation reaction (alkaline phosphatase + BCIP-NTB)	5–6 h
[22]	IT	*A. baumannii*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *C. koseri*, *C. rodentium*, *C. difficile*, *C. kluyveri*, *C. perfringens*, *C. diphtheriae*, *C. efficiens*, *C. jeikeium*, *E. faecalis*, *E. coli*, *E. fergusonii*, *H. influenza*, *K. pneumonia*, *L. innocua*, *L. monocytogenes*, *M. luteus*, *O. anthropic*, *P. acnes*, *P. mirabilis*, *P. aeruginosa*, *P. fluorescens*, *P. mendocina*, *P. putida*, *S. enterica*, *S. aureus*, *S. carnosus*, *S.epidermidis*, *S. haemolyticus*, *S. lugdunensis*, *S. saprophyticus*, *S. maltophilia*, *S. equi*, *S. pyogenes*	Reference strains, clinical isolates, and two clinical saliva samples	DNA extraction, gDNA fragmentation (95 °C for 3 min), chemical labeling with Cy3	Microarray hybridization, fluorescence detection	n/a

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Table 4.1 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[15]	DE	*Chlamydia* spp., *Chlamydophila* spp.	282 animal and 11 human samples (nasal and conjunctival swabs, formalin-fixed, paraffin-embedded and fresh organ tissue, milk, feces, and cell culture)	DNA extraction, PCR (biotinylated primer)	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	24 h
[23]	BE	*E. faecium*, *S. aureus*, *S. pneumoniae*, *A. calcoaceticus*, *B. cepacia*, *C. freundii*, *E. aerogenes*, *E. cloacae*, *E. coli*, *H. influenza*, *K. pneumoniae*, *L. pneumophila*, *M. catarrhalis*, *M. tuberculosis*, *P. aeruginosa*, *P. mirabilis*, *P. stuartii*, *P. vulgaris*, *S. maltophilia*, *S. marcescens*, *A. flavus*, *A. fumigatus*, *C. albicans*	200 clinical isolates, 25 endotracheal aspirates, and sputum samples	DNA extraction, multiplex-PCR (fus/rps gene complex, gyrA gene)	Hybridization, Silverquant labeling (Eppendorf)	n/a
[24]	DE	*S. aureus*, *E. coli*, *P. aeruginosa*	43 clinical isolates, 13 positive blood cultures	DNA extraction, no PCR, genomic DNA chemically labeled with fluorophores	Hybridization with Tecan HS400 hybridization station (17 h) on microarrays with 200–800 bp long probes	>24 h

[25]	FR	*A. caviae*, *A. butzleri*, *C. coli*, *C. fetus*, *C. jejuni*, *C. upsaliensis*, *E. coli (EHEC, EIEC*, *EPEC*, and *STEC)*, *G. hollisae*, *K. oxytoca*, *L. monocytogenes*, *S. enterica*, *S. sonnei*, *T. whipplei*, *V. alginolyticus*, *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *Y. enterocolitica*, *G. obscuriglobus*, *P. staleyi*, *P. brasiliensis/maris*, *P. limnophilus*, *R. baltica* + 7 enteropathogenic viruses	40 pathological stool specimens	Diarrheal stools were lyophilized, vortexed with glass beads, specimen were ground using the FastPrep (MP Bio-medicals) apparatus, followed by lysis with the Nucleospin Tissue kit (Macherey Nagel) and DNA extraction using the Qiagen EZ1 DNA Tissue kit	Extracted genomic DNA was Cy3-labeled with the ULS Labeling Kit and hybridized on the microarray for 40 h	>48 h
[26]	CN	*Y. enterocolitica*, *S.* spp., *S. typhi*, *Brucella* spp., *V. cholerae*, and *E. coli O157:H7*	362 clinical specimens (rectal swabs)	DNA extraction, multiplex PCR with biotinylated primer	DNA microarray hybridization + combined with tyramide signal amplification	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis
[26]	CN	*Y. enterocolitica*, *Salmonella* spp., *S. typhi*, *Brucella* spp., *V. cholerae*, *E. coli*	92 reference strains, 362 clinical samples (swabs)	DNA extraction, multiplex PCR	Low density DNA microarray, hybridization (1 h), enzymatic signal amplification (MRP + tyramide-signal-amplification), fluorescence detection	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis

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Table 4.1 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[27]	KR	*Enterococcus* spp., *Listeria* spp., *Staphylococcus* spp., *Streptococcus* spp., *Bacteroides* spp., *E. coli*, *Enterobacter/Klebsiella* spp., *Hemophilus* spp., *Pseudomonas* spp., *S.* spp., *E. faecalis*, *E. faecium*, *E. avium*, *L. monocytogenes*, *L. ivanovii*, *L. grayi*, *L. welshimeri*, *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *S. bovis*, *B. fragilis*, *B. thetaiotaomicron*, *B. ovatus*, *E. cloacae*, *E. aerogenes*, *E. agglomerans*, *K. pneumoniae*, *K. oxytoca*, *H. influenzae*, *H. ducreyi*, *P. aeruginosa*, *P. stutzeri*, *S. marcescens*, *S. grimesii*, *S. entomophila*	825 positive blood cultures	DNA extraction (InstaGene Matrix Kit), PCR with universal, biotinylated primer	30 min hybridization in the presence of Cy5-streptavidin on microarrays with genus and species-specific probes targeting the bacterial ITS of the 16S-23S rRNA	24 h
[28]	KR	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, *S. enterica serovar Typhimurium*, *S. enterica serovar Enteritidis*, *S. flexneri*, *S. sonnei*, *enterotoxigenic E. coli (ETEC)*, *enterohemorrhagic E. coli (EHEC)*, *Y. enterocolitica*	82 stool samples from patients with food-borne enteritis	DNA extraction (AccuPower Stool DNA extraction kit, Bioneer)	Multiplexed PCR of bacterial virulence-factor genes, chemically labeling (BioPrime Array CGH Genomic Labeling System, Invitrogen) + hybridization (4 h)	12 h

[29]	CN	*C. difficile*, *C. jejuni*, *C. perfringens*, *V. parahaemolyticus*, *P.* spp., *L. monocytogenes*, *B. cereus*, *Y. enterocolitica*, *E. faecalis*, *P. aeruginosa*, *S. aureus*, *E. coli*, *Shigella* spp., and *Salmonella* spp.	1500 diarrheal stool samples + 200 stool samples of healthy individuals	DNA extraction (Qiagen QIAamp DNA Stool extraction kit), PCR with 16S rRNA primer + 2 virulence gene specific primer pairs	1 h hybridization of Cy3-labeled PCR products in humidification chamber without mixing	5 h
[30]	JP	*S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. intermedius*, *S. saprophyticus*, *S. warneri*, *S. pneumonia*, *S. pyogenes*, *S. agalactiae*, *S. bovis*, *S. canis*, *S. equisimilis*, *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. mitis*, *S. oralis*, *S. sanguinis*, *A. viridans*, *G. morbillorum*, *M. luteus*, *B. cereus*, *B. subtilis*, *C. jeikeium*, *C. aquaticum*, *L. monocytogenes*, *E. coli*, *K. pneumonia*, *K. oxytoca*, *S. marcescens*, *S. Typhi Paratyphi A*, *E. cloacae*, *E. aerogenes*, *P. agglomerans*, *C. freundii*, *C. koseri*, *M. morganii*, *P. mirabilis*, *P. stuartii*, *P. aeruginosa*, *B. cepacia*, *S. maltophilia*, *A. calcoaceticus*, *A. faecalis*, *A. xylosoxidans*, *C. indologenes*, *C. meningosepticum*, *C. acidovorans*, *H. influenza*, *M. catarrhalis*, *N. meningitides*, *A. hydrophila*, *E. corrodens*, *A. actinomycetemcomitans*, *L. pneumophila*, *B. fragilis*, *P. melaninogenica*, *F. nucleatum*, *V. parvula*, *C. perfringens*, *E. lentum*, *P. acnes*, *L. acidophilus*, *P. magnus*, *E. faecalis*, *E. faecium E. avium*, *E. casseliflavus*, *E. gallinarum*, *M. gordonae*, *M. kansasii*	335 cultures from patients with hematological diseases	Enrichment culture, DNA extraction, PCR with universal 16S rDNA primer (Cy5-labeled)	Hybridization for 1 h	24 h

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Table 4.1 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[31]	DE	*Acinetobacter* spp., *E. cloacae*, *P. aeruginosa*, *P. mirabilis*, *K. pneumonia*, *K. oxytoca*, *E. coli*, *S. agalactiae*, *S. epidermidis*, *S. aureus*, *E. faecium*, *E. faecalis*, *Stenotrophomonas* spp., *C. albicans*, *S. pyrogenes*, *S. pneumoniae*, *S. viridans*	9 reference strains + 3 swab samples from superficial wounds	DNA extraction, multiplex PCR (800 primer pairs), labeling PCR	Hybridization (18 h) in HS400 TECAN automated hybridization station, fluorescence detection	>24 h
[10]	KR	*M. tuberculosis*, *M. avium-intracellulare complex (MAC)*, *M. fortuitum*, *M. chelonae*, *M. abscessus*, *M. kansasii*, *M. gordonae*, *M. scrofulaceum*, *M. szulgai*, *M. vaccae*, *M. xenopi*, *M. terrae*, *M. flavescens*, *M. smegmatis*, *M. malmoense*, *M. simiae*, *M. marinum*, *M. ulcerans*, *M. gastri*, and *M. leprae*	46 reference strains, 149 clinical isolates, 155 clinical sputum samples	DNA extraction, ITS PCR	Hybridization (30 min), fluorescence detection	4.5 h
[32]	DE	*B. mallei*, *B. pseudomallei*	Reference strains	DNA extraction, multiplex-PCR (biotinylated primer)	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	n/a
[33]	CN	*E. coli O157:H7*, *V. cholerae*, *V. parahaemolyticus*, *S.* spp., *S. aureus*, *Rotavirus Norwalk virus*	86 samples isolated from patients, fishery products, or environment	Enrichment culture, DNA extraction, multiplex PCR	Hybridization with biotinylated PCR products, streptavidin-coated magnetic bead incubation, readout by eye, CCD camera	13 h

[34]	AT	*A. baumannii*, *A. johnsonii*, *A. Iwoffii*, *A. radioresistens*, *C. freundii*, *C. koseri*, *E. cloacae*, *E. aerogenes*, *K. pneumoniae*, *K. oxytoca*, *E. coli*, *M. morganii*, *P. mirabilis*, *P. vulgaris*, *P. aerogenes*, *S. marcescens*, *S. maltophilia*, *S. pneumoniae*, *S. pyrogenes*, *E. faecium*, *Stapylococcus* spp., *S. aureus*, *S. epidermidis*, *E. faecalis*, *C. albicans*, *C. parapsilosis*	Reference strains and clinical isolates (blood)	DNA extraction, multiplex PCR (16S rRNA), fluorescence labeling via primer extension	Hybridization (1 h), fluorescence detection	6 h
[35]	KR	*A. baumannii*, *A. succiniproducens*, *B. cepacia*, *B. catarrhalis*, *B. ovatus*, *B. vulgatus*, *B. fragilis*, *C. hominis*, *E. meningoseptica*, *C. acidovorans*, *E. cloacae*, *K. oxytoca*, *C. freundii*, *E. aerogenes*, *M. morganii*, *K. pneumoniae*, *E. coli*, *S. paratyphimurium*, *K. kingae*, *E. faecalis*, *E. faecium*, *O. anthropi*, *N. meningitides*, *N. gonorrhoeae*, *F. necrophorum*, *A. actinomycetemcomitans*, *L. monocytogenes*, *S. mutans*, *L. pneumophila*, *P. aeruginosa*, *P. anaerobius*, *P. mirabilis*, *P. vulgaris*, *S. maltophilia*, *S. marcescens*, *S. epidermidis*, *S. aureus*, *S. agalactiae*, *S. bovis*, *S. sanguis*, *S. anginosus*, *S. pneumoniae*, *V. cholerae*, *V. vulnificus*, *C. neoformans*, *C. glabrata*, *C. krusei*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*	Reference strains + positive blood culture samples	DNA extraction, PCR with universal primer in two separate tubes, lambda exonuclease treatment to generate single stranded products	6 h hybridization on microarrays with probes targeting the bacterial 23S rRNA, 16S–23S rRNA ITS, and the fungal 18S rRNA	10 h (after positive blood culture)
[36]	CN	*E. coli*, *V. cholerae*, *V. parahaemolyticus*, *S. enterica*, *C. jejuni*, *Shigella* spp., *Y. enterocolitica*, *L. monocytogenes*	34 stool specimens	DNA extraction, multiplex-PCR with Cy5-dUTP	12 h hybridization with GenTAC hybridization station (Genomic Solutions)	15 h

ITS: internal transcribed spacer region; CC: country code.

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200–800 bp long PCR product probes on a medium-density microarray. This approach enabled the detection of sepsis-causing pathogens as well as several virulence factors and antibiotic resistance genes down to 1 ng of target DNA [31]. Previously, this research group from the University of Cologne used the same type of microarray for direct PCR-free detection of *S. aureus*, *E. coli*, and *Pseudomonas aeruginosa* from positive blood cultures [24, 38]. Yoo *et al.* developed a DNA microarray for the detection of 50 bacterial and 7 fungal species [35]. The probes were directed against the bacterial 23S rRNA, the 16S–23S rRNA ITS (internal transcribed spacer), and the fungal 18S rRNA. The authors tested 112 blood cultures with a sensitivity of 93% and an LOD of 10 cfu. A DNA microarray for the detection 30 bacteria associated with sepsis contained 1 universal, 3 Gram stain-specific, 9 genus-specific, and 30 species-specific probes directed against the 16S–23S rRNA ITS region. The microarray was validated with 825 positive blood culture samples from which 708 (85.8%) were identified correctly by the oligonucleotide chip [27]. Negoro *et al.* [30] developed a DNA microarray which covered 72 bacteria which are often identified in positive blood cultures in combination with a PCR using universal PCR primers to amplify a variable region of the 16S rDNA. The microarray method was validated with 335 blood specimens from 99 patients with hematological diseases who had febrile illness. The sensitivity and specificity of the microarray test compared with conventional blood culture testing were 92.7 and 99.3%, respectively.

The "Prove-it Sepsis" assay from Mobidiag (Helsinki, Finland) combined amplification and detection of the *gyrB*, *parE*, and *mecA* genes for the identification of 50 Gram-positive and Gram-negative bacteria in positive blood cultures [39]. The assay uses the Array Strip platform developed by Clondiag in Jena. The company was acquired by Alere in 2006. The microarray detection principle is based on the formation of a precipitating product from a horseradish peroxidase-catalyzed substrate reaction. The enzyme is bound to the hybridized PCR product via streptavidin–biotin interaction. The arrays are spotted on the bottom of microtiter plate wells. A large-scale study of 2107 positive blood-culture samples of 3318 blood samples from patients with clinically suspected sepsis investigated by the conventional culture method and the "Prove-it Sepsis" assay revealed a sensitivity of 95% and a specificity of 98% of the microarray method. This study also compared the mean turnaround time of the microarray method and the conventional culture method, revealing an average time advantage of 18 h for the microarray. The disadvantage of this sepsis test was that it still required an enrichment of blood culture and was not sensitive enough to detect bacteria directly from patient blood samples.

4.2.1.2 **Pneumonia**

Pneumonia is a serious life-threatening condition which may be conceived in the community or in the healthcare environment. As the etiology of pneumonia is difficult to identify and rapid therapy initiation is of prime importance, DNA microarrays are a promising platform to develop commercial diagnostic assays. As an example, Eppendorf Array Technologies in Namur (Belgium) developed

a real-time Array-PCR platform for infectious disease diagnostics which combined the characteristics of real-time PCR with the multiplexing capability of microarray detection. A chip for the diagnosis of ventilator-associated pneumonia (VAP)chip covered 13 Gram-positive and Gram-negative bacteria and 24 antibiotic resistance genes that mediate resistance to beta-lactam agents, including extended-spectrum cephalosporins and carbapenems and *mecA* [40]. The VAPChip was validated with 292 reference strains and clinical isolates, showing 98.6% sensitivity and 100% specificity for species identification. Regarding the detection of the resistance genes, the system presented a global sensitivity and specificity of 98.7% and 97.7%, respectively. The LOD of the test was between 10 and 100 genome copies/PCR. The VAPChip does not appear to be commercially available. In contrast, Curetis AG developed and launched a diagnostic platform, the Unyvero System, which is aimed at decentralized diagnostics (see Table 4.2). The Unyvero System comprises sampling tubes, a lysator device, a cartridge with preloaded reagents, and a reader. The cartridge contains special departments for all analytical steps (DNA purification, amplification, and detection by hybridization on a membrane microarray with colorimetric detection). Once the cartridge is loaded into the analyzer, all sample processing happens automatically without any further operator interaction. The cartridge is a closed system, and any possibility of spilling sample or reagents as well as contamination risks are minimized. One of the products developed is the Pneumonia Cartridge, which analyzes the antibiotic resistance markers simultaneously with the pathogen detection. The Unyvero Pneumonia Cartridge determines 39 targets involving beta-lactam-resistance, including ESBL (extended-spectrum beta-lactamase) and KPC (*Klebsiella pneumoniae* carbapenemase), macrolide resistance, quinolone resistance, multidrug resistance, and the most clinically relevant pathogens in approximately 4 h.

4.2.1.3 Infectious Diarrhea

Infectious diarrhea is one of the major public health problems worldwide. It accounted for nearly 10% of the 7.6 million deaths in children younger than 5 years in 2010 [37]. Several research groups developed DNA microarray tests for the detection of enteropathogenic bacteria directly from human diarrheal stool samples as an alternative to traditional culture-based methods. Donatin *et al.* [25] developed a DNA microarray for the detection of 33 enteropathogenic bacteria and 7 enteropathogenic viruses. Genomic DNA extracted from stool samples was fluorescence-labeled and hybridized on the microarray without prior amplification, but with a rather long hybridization time of 40 h. The microarray was tested with 40 pathological stool specimens, showing an overall specificity of 100% and an overall sensitivity of 97.5%. Jin *et al.* [26] developed an assay using DNA microarray coupled with multiplex PCR and TSA-Cy3 labeling for the simultaneous identification of six species of human diarrheal bacteria with an LOD of 10^3 cfu/ml [26]. Tests of 362 anus swab samples collected from an epidemic of *C. cholera* revealed a specificity of 98.6%. You *et al.* combined a multiplex PCR of virulence gene targets of eight enteropathogenic bacteria with

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Table 4.2 Commercial DNA microarray assays for pathogen detection[a)] .

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Alere Technologies GmbH www.alere-technologies.com	*S. aureus* Genotyping Kit 2.0	DE	*S. aureus* (MRSA/MSSA, 336 genetic markers)	Colonies (pure and clonal)	No	Microarrays, reagents, positive control	7–8 h (hands-on-time 80 min)	RUO
Alere Technologies GmbH	Influenza A Genotyping Kit 96	DE	Influenza A virus (200 markers for H- and N-typing)	Field samples (e.g., swabs)	No	Microarrays, reagents	7–8 h (hands-on-time 80 min)	RUO
Alere Technologies GmbH	*E. coli* Genotyping Kit	DE	92 virulence genes/alleles	Colonies (pure and clonal)	No	Microarrays, reagents	7–8 h (hands-on-time 110 min)	RUO
Alere Technologies GmbH	AMR-ve Genotyping Kit	DE	54 resistance genes of gram-bacteria	Colonies (pure and clonal)	No	Microarrays, reagents, positive control	7–8 h (hands-on-time 2 h)	RUO
Alere Technologies GmbH	*P. aeruginosa* 2 Genotyping Kit	DE	99 probes for different genetic markers	Colonies (pure and clonal)	No	Microarrays, reagents	7–8 h (hands-on-time 2 h)	RUO

Alere Technologies GmbH	Salmonella SeroGenotyping Kit	DE	132 different *Salmonella* serovars, including genetic markers for 28 O-antigens, 86 H-antigens and 62 antibiotic resistance genes	Colonies (pure and clonal)	No	Modular: microarrays and reagents	7–8 h (hands-on-time 2 h)	RUO
Alere Technologies GmbH	*E. coli* stx (Shiga-Toxin)	DE	70 genetic markers to identify all subtypes of Shiga-Toxin 1 and 2	Colonies (pure and clonal)	No	Modular: microarrays and reagents	7–8 h (hands-on-time 2 h)	DEV
Alere Technologies GmbH	*E. coli* H&O typing	DE	210 genetic markers to identify 50 O-antigens and 59 H-antigens	Colonies (pure and clonal)	No	Modular: microarrays and reagents	7–8 h (hands-on-time 2 h)	DEV
Alere Technologies GmbH	Carbapenemase typing	DE	80 genetic markers to identify resistance genes for ESBLs and Carbapenemases, 15 species markers for Enterobacteriaceae *K. pneumoniae*, *P. aeruginosa*, *E. coli*, *A. baumannii*, *C. freundii*	Colonies (pure and clonal)	No	Modular: microarrays and reagents	7–8 h (hands-on-time 2 h)	DEV

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
AutoGenomics	INFINITI Respiratory Viral Plus	US	Influenza (A/Swine H1N1 and B), human parainfluenza virus (HPIV 1, 2, 3, 4), rhinovirus A and B, enterovirus (A, B, C, D), coronavirus (HKU1, OC43, NL63, 229E), human metapneumovirus (HMPV A and B), human respiratory syncytial virus (HRSV A and B), adenovirus A, B, C, E	8 µl of purified RNA/ reaction	No, auto-mated workflow	BioFilmChip microarray and Intellipac Reagent Module	3 h+	CE
www.auto-genomics.com								
AutoGenomics	INFINITI FLU A-sH1N1	US	Influenza A-swine H1N1	8 µl of purified RNA/ reaction	No, auto-mated workflow	BioFilmChip microarray and Intellipac Reagent Module	3 h+	CE

AutoGenomics	• INFINITI HPV Genotyping • INFINITI HPV-HR QUAD • INFINITI HPV-QUAD	US	Human papillomavirus, subtypes	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	CE
AutoGenomics	• INFINITI STD-6 Panel • INFINITI STD-6 QUAD	US	*C. trachomatis*, *N. gonorrhoeae*, *U. urealyticum*, *M. genitalium*, *M. hominis*, *T. vaginalis*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO
AutoGenomics	INFINITI CT/NG QUAD	US	*C. trachomatis*, *N. gonorrhoeae*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO
AutoGenomics	INFINITI Leuko QUAD	US	*C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO
AutoGenomics	INFINITI UroGen QUAD	US	*U. urealyticum*, *M. genitalium*, *M. hominis*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	CE
AutoGenomics	INFINITI UroGen Plus QUAD	US	*U. urealyticu*, *U. parvum*, *M. genitalium*, *M. hominis*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
AutoGenomics	INFINITI Candida Vaginitis QUAD	US	*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. tropicalis*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	CE
AutoGenomics	INFINITI MDR-TB	US	*M. tuberculosis rpoB*, *katG*, *inhA*, *pncA*, *M. bovis*	Decont-aminated cell lysate (direct from sediment without DNA extraction)	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO
AutoGenomics	INFINITI NTM Panel	US	*M. genus*, *M. avium complex (M. avium*, *M. Intracellulares)*, *M. kansasii*, *M. gordonae*, *M. scrofulaceum*, *M. xenopi*, *M. marinum/M. ulcerans*, *M. haemophilum*, *M. fortuitum*, *M. chelonae*, *M. abscessus/M. mucogenicum*, *M. smegmatis*	n/a	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	RUO

AutoGenomics	INFINITI Resolve QUAD	US	*G. vaginalis*, *C. albicans*,*C. glabrata*, *T. vaginalis*, *Mobiluncus* spp. *(mulieris* and *curtisii)*	50 ng DNA/ reaction	No, auto-mated workflow	BioFilmChip microarray, Intellipac Reagent Module and Amp Mix	3 h+	CE
Capitalbio www.capi-talbio.com	Mycobacteria Resistance Array Kit	CN	14 mutations in three genes associated with resistance to rifampicin and isoniazid	Clinical isolates or clinical sputum samples	No	Sample preparation kit, PCR reagents, microarray slides	na	CE
Capitalbio	Mycobacteria Identification Array Kit	CN	*M. tuberculosis*, *M. Kansassi*, *M. chelonae*, *M. smegmatis*, *M. scrofulaceum*, *M. fortuitum*, *M. gordonae*, *M. avium*, *M. phlei*, *M. xenopi*, *M. marion*	Clinical isolates or clinical sputum samples	No	Sample preparation kit, PCR reagents, microarray slides	na	CE
Check Points	Check-MDR CT101	NL	Carbapenemases (KPC, NDM), ESBLs (CTX-M, TEM, SHV), AmpCs (CMY, DHA, FOX, MOX, ACC, MIR, ACT)	Colonies (bacterial culture)	No	Ligation-mediated PCR reagents, Check-Points ArrayTube microarrays, detection buffer, blocking buffer, cunjugate solution, staining solution	6.5 h (excluding extrac-tion)	CE

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.check-points.nl								RUO (US)
Check Points	Check-MDR CT102	NL	Carbapenemases (KPC, NDM, VIM, IMP, OXA-48), ESBLs (CTX-M, TEM, SHV)	Colonies (bacterial culture)	No	Ligation-mediated PCR reagents, Check-Points ArrayTube microarrays, detection buffer, blocking buffer, cunjugate solution, staining solution	6.5 h (excluding extraction)	CE
								RUO (US)
Check Points	Check-MDR CT103	NL	Carbapenemases (KPC, NDM, VIM, IMP, OXA-48), ESBLs (CTX-M, TEM, SHV), AmpCs (CMY, DHA, FOX, MOX, ACC, MIR, ACT)	Colonies (bacterial culture)	No	Ligation-mediated PCR reagents, Check-Points ArrayTube microarrays, detection buffer, blocking buffer, cunjugate solution, staining solution	6.5 h (excluding extraction)	CE
								RUO (US)

Chipron GmbH www.chipron.com	BAC Dent 2.4	DE	*P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *T. denticola*, *T. forsythensis*, *F.* sp., *E. corrodens*, *C. ginivalis*, *P. micros*, *E. nodatum*, *C. rectus*	n/a	No	PCR-primer, LCD-arrays, hybridization buffer, label, dilution buffer, modulator, stain, wash buffer, pattern file, manual, MSDS	2.5 h (excluding extraction)	CE
Chipron GmbH	HPV 3.5	DE	Detection and typing of 32 HPV types (low and high risk): HPV 06, 11, 16, 18, 31, 33, 35, 39, 42, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 67, 68, 70, 72, 73, 81, 82, 83, 84, 90, 91	n/a	No	PCR-primer, LCD-arrays, hybridization buffer, label, dilution buffer, modulator, stain, wash buffer, pattern file, manual, MSDS	3.5 h (excluding extraction)	RUO
Chipron GmbH	Fungi 2.1	DE	*A. flavus*, *A. fumigatus*, *A. versicolor*, *A. nidulans*, *A. niger*, *A. terreus*, *C. albicans*, *C. dubliniensis*, *C. glabrata*, *C. guilliermondii*, *C. kefyr*, *C. krusei*, *C. lambica*, *C. lusitaniae*, *C. parapsilosis*, *C. pelliculosa*, *C. tropicalis*, *Mucor* spp., *Rhizomucor* spp., *C. neoformans*, *P. variotii*, *S. prolificans*, *A. corymbifera*	n/a	No	PCR-primer, LCD-arrays, hybridization buffer, label, dilution buffer, modulator, stain, wash buffer, pattern file, manual, MSDS	3 h (excluding extraction)	RUO

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Chipron GmbH	Dengue 1.4	DE	Detection and typing of dengue virus 1, 2, 3, 4	n/a	No	PCR-primer, LCD-arrays, hybridization buffer, label, dilution buffer, modulator, stain, wash buffer, pattern file, manual, MSDS	3.5 h (excluding extraction)	RUO
Chipron GmbH	MYCO Direct 1.7	DE	*M. tuberculosis* (complex), *M. avium*, *M. intracellulare*, *M. gordonae*, *M. kansasii*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. celatum*, *M. haemophilum*, *M. heckeshornense*, *M. abscessus*, *M. chelonae*, *M. fortuitum* (complex), *M. peregrinum*	n/a	No	PCR-primer, LCD-arrays, hybridization buffer, label, dilution buffer, modulator, stain, wash buffer, pattern file, manual, MSDS	2.5 h (excluding extraction)	RUO
Clondiag/Alere	ArrayTube/ Arraystrip	DE	OEM manufacturer for companies using the ArrayTube platform	n/a	No	ArrayTube microarray	n/a	n/a

www.clondiag.com								
Curetis www.curetis.com	Unyvero P50	DE	Species: *A. baumannii*, *C. pneumoniae*, *Enterobacter* sp., *E. coli*, *H. influenzae*, *K. pneumoniae*, *K. oxytoca*, *L. pneumophila*, *M. catarrhalis*, *M. morganii*, *P. jirovecii*, *P.* sp., *P. aeruginosa*, *S. marcescens*, *S. aureus*, *S. maltophilia*, *S. pneumoniae* Antibiotic resistance: *ctx-M*, *dha*, *ebc*, *ermA*, *ermB*, *ermC*, *gyrA83*, *gyrA87*, *int1*, *kpc*, *mecA*, *mefA/E*, *msrA*, *oxa51 like*, *parC*, *shv*, *sul1*, *tem*	Unprocessed primary respiratory samples: sputum, tracheal aspirate, tracheal lavage, bronchial aspirate, bronchial lavage	Yes (Unyvero Lysator + Unyvero Cartridge)	P50 Cartridge, Sample Handling Tool, Sample Tube, Sample Tube Cap, Mastermix	Approxi-mately 4.5 h	CE
Curetis	Unyvero i60 ITI	DE	>80 pathogen species and >20 antibiotic resistance markers relevant in implant and tissue infections	Primary orthopedic and chirurgic samples: sonification fluid, synovial fluid, pus, swabs, biopsy	Yes (Unyvero Lysator plus Unyvero Cartridge)	i60 Cartridge, Sample Tube, Sample Tube Cap, Master mix	<5 h	DEV, launch end 2013

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Curetis	Blood Stream Infection Cartridge	DE	Not specified	—	n/a	—	n/a	Planned
Curetis	Tuberculosis cartridge	DE	Not specified	—	n/a	—	n/a	Planned
Dr Chip www.bio-drchip.com.tw	DR. EV IVD Kit	TW	Pan-enterovirus, enterovirus 71, coxsackie A16	Throat swab, rectal swab, feces, nasopharyngeal aspirate, serum, and cerebral spinal fluid	No	Reagents for RNA extraction, reverse transcription (RT), cDNA amplification, polymer chip hybridization, colorimetric detection	6 h	RUO
Dr Chip	DR. RV IVD Kit	TW	Severe acute respiratory syndrome (SARS coronavirus), influenza A, influenza B, parainfluenza I, parainfluenza II, parainfluenza III, RSV, adenovirus	Throat swab in transport medium or nasopharyngeal aspirate	No	Reagents for viral RNA/DNA extraction, reverse transcription (RT), PCR, polymer chip hybridization, colorimetric detection	6 h	RUO
Dr Chip	DR. Food Kit	TW	*S. aureus*, *E. coli*, *Y. enterocolitica*, *B. cereus*, *C. perfringens*, *L. monocytogenes*, *S.* spp., *S.* spp., *Vibrio* spp.	Food samples	No	n/a	6 h	n/a

Dr Chip	Dr. Milk Kit	TW	*Streptococcus* spp., *S. agalactiae*, *S. bovis*, *S. dysgalactiae*, *S. uberis*, *E. coli*, *S. aureus*	Milk samples	No	n/a	6 h	n/a
Gene In www.genein.com	CombiChip Mycobacteria Genotyping DNA Chip	KR	Panmycobacteria, *M. vaccae*, *M. tuberculosis complex*, *M. xenopi*, *M. avium-intracellulare complex*, *M. terrae*, *M. fortuitum*, *M. flavescens*, *M. chelonae*, *M. smegmatis*, *M. abscessus*, *M. malmoense*, *M. kansasii*, *M. simiae*, *M. gordonae*, *M. marinum-ulcerance*, *M. scrofulaceum*, *M. gastri*, *M. szulgai*, *M. leprae*	n/a	No	n/a	n/a	n/a
Genomica	CLART HPV2	ES	Human papillomavirus, (6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 43, 44, 45, 51, 52, 53, 54, 56, 58, 59, 61, 62, 66, 68, 70, 71, 72, 73, 81, 82, 83, 84, 85 y 89)	Liquid cytology, swabs, and paraffin embedded tissues	No	Reagents for multiplex PCR to generate biotin-labeled products; detection on low density microarrays via streptavidin–peroxidase catalyzed preceipitaion reaction	8 h	CE

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.genomica.es								
Genomica	CLART PneumoVir	ES	Adenovirus; bocavirus; coronavirus; enterovirus (echovirus); influenza virus A (subtypes H3N2 human, H1N1 human, B, C, and H1N1/2009); metapneumovirus (subtypes A and B); parainfluenza virus 1, 2, 3, and 4 (subtypes A y B); rhinovirus; respiratory syncitial virus type A (VSR-A); respiratory syncitial virus type B (VSR-B)	Nasopharyngeal wash, nasopharyngeal exudate, bronchoalveolar lavage	No	Extraction–purification reagents, amplification reagents (RT, multiplex-PCR), and visualization reagents (including microarrays)	Hours	CE
Genomica	CLART ENTHERPEX	ES	HSV-1, HSV-2, VZV, CMV, EBV, HHV-6, HHV-7, and HHV-8; poliovirus, echovirus, coxsackievirus	Swabs, serum, plasma, cerebrospinal fluidi, biopsies	No	Viral genome-specific fragment amplification via multiple RT-PCR, subsequent detection via hybridization with microorganism-specific binding probe arrays	8 h	n/a

Genomica	CLART EnteroBac	ES	*Salmonella* spp. (all the described species), *Shigella* spp. (*S. dysenteriae*, *S. sonnei*, *S. boydii*, and *S. flexneri*), *Y. enterocolitica*, *Y.* spp. (*Y. pestis*, *Y. pseudotuberculosis*, *Y. enterocolitica*), *C.* spp. (*C. lari*, *C. laridis*, *C. upsaliensis*, *C. jejuni*, *C. coli*), *C. jejuni*, *C. coli*, *E. coli enteropathogenic EPEC*: (*E. coli enterohemoragic*, *E. coli*, *enteroinvasive*, *E. coli*, *enterotoxigenic and E. coli enteropathogenic*), *C. difficile B*, *Aeromonas* spp., producers of aerolisin	Stool samples	No	Extraction–purification reagents (including extraction control), amplification reagents (multiplex-PCR), and visualization reagents (including microarrays)	Hours	CE
Genomica	CLART STIs A	ES	*C. trachomatis*, *subtypes*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis*	Urine, swabs (cervical, vaginal, urethral, and rectal)	No	Amplification and visualization reagents (including CLART eight-well strip microarrays)	Hours	CE
Genomica	CLART STIs B	ES	*U. urealyticum/parvum*, *M. hominis C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. krusei*, *C. tropicalis*, *C. guilliermondii*, *C. dubliniensis*, *Treponema pallidum*, *H. ducreyi*, herpes virus types I and II	Urine, swabs (cervical, vaginal, urethral, and rectal)	No	Amplification and visualization reagents (including CLART eight-well strip microarrays)	Hours	CE

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Genomica	CLART SeptiBac	ES	*S. aureus*, *S. epidermidis* *S. hominis*, *S. haemolyticus*, *S. pyogenes/dysgalactiae*, *S. pneumoniae*, *S. mitis*, *S. agalactiae*, *S. sanguinis/parasanguinis*, *Streptococcus de grupo milleri (including S. constellatus y S. anginosus)*, *Detección genérica para Streptococcus* spp., *E. faecium*, *E. faecalis*, *Gram positive cocci (CGPs)*, *L. monocytogenes*, *C. perfringens*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *K.* spp. *(K. pneumoniae, K, oxytoca)*, *S. enteric*, *E. cloacae*, *E. aerogenes*, *Enterobacter* spp. *(E. cloacae, E. aerogenes)*, *C. freundii*, *C.* spp. *(C. freundii, C. koserii)*, *S. marcescens/plimutica P. vulgaris/penneri*, *P. mirabilis*,	Positive blood cultures	No	Amplification and visualization reagents (including CLART eight-well strip microarrays)	Hours	CE

			P. spp. *(P. vulgaris, P. mirabilis, P. penneri), H. influenza, H.* spp. *(H. influenzae, H. parainfluenzae), A. baumannii, B. fragilis, B.* spp. *(B. fragilis, B. faecis), Pseudomonas* spp. *(P. aeruginosa, P. putida, P. stuartii), S. maltophilia, C. albicans, C. glabrata, C. krusei*					
Great Basin	Staph ID/R	US	*S. aureus, Staphylococcus* species, *mecA*	Positive blood cultures	Yes	Catridge containing reagents for multiplex helicase-dependet amplification, microarray, detection based on HRP catalyzed precipitation reaction	1.5 h	DEV
www.gbscience.com								
Great Basin	*C. difficile*	US	*C. difficile, tcdA, tcdB*	—	Yes	Catridge containing reagents for multiplex helicase-dependet amplification, microarray, detection based on HRP catalyzed precipitation reaction	n/a	510(k)/CE

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Great Basin	Nasal MRSA	US	*S. aureus*, *mecA*	Nasal or perianal swabs	Yes	Transfer solution for nasal or perianal swabs + Catridge containing reagents for multiplex helicase-dependet amplification, microarray, detection based on HRP catalyzed precipitation reaction	n/a	DEV
Great Basin	Fungal pathogens	US	*Candida* species	Positive blood culture	Yes	Catridge containing reagents for multiplex helicase-dependet amplification, microarray, detection based on HRP catalyzed precipitation reaction		DEV

Greiner	CytoInspect	DE	*A. axanthum*, *A. laidlawii*, *A. modicum*, *A. morum*, *A. oculi*, *A. vituli*, *M. arginini*, *M. alkalescens*, *M. arthritidis*, *M. bovigenitalium*, *M. bovirhinis*, *M. bovis*, *M. bovoculi*, *M. buccale*, *M. californicum*, *M. canadense*, *M. canis*, *M. quirhinis*, *M. faucium*, *M. fermentans*, *M. flocculare*, *M. gallinaceum*, *M. gallinarum*, *M. gallisepticum*, *M. genitalium*, *M. glycophilum*, *M. hominis*, *M. hyopharyngis*, *M. hyopneumoniae*, *M. hyorhinis*, *M. Hyosynoviae*, *M. orale*, *M. pirum*, *M. pneumoniae*, *M. ulmonis*, *M. salivarium*, *M. synoviae*, *S. citri*, *S. kunkelii*, *U. diversum*	Cell cultures and other biological materials	No	DNA extraction kit and test kit containing PCR reagents, internal control, and microarrays	5 h	n/a

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.greinerbioone.com								
Greiner	ParoCheck	DE	*P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia*, *F. nucleatum*, *E. corrodens*, *T. denticola*, *A. viscosus*, *T. forsythensis*, *C. rectus*, *P. micros*, *S. mitis*, *S. gordonii*, *P. nigrescens*, *S. constellatus*, *V. parvula*, *C. ginivalis*, *A. odontolyticus*, *C. concisus*, *E. nodatum*, *C. gracilis*	Gingival swab samples	No	Specimen collection kit + PCR mastermix, microarray slides, hybridization buffer, washing buffer	2.5 h	CE
Greiner	PapilloCheck	DE	24 Human papillomavirus types (6, 1, 40, 42, 43, 44; 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 70, 73, 82)	Human cervical samples	No	PapilloCheck collection kit + detection kit containing PCR reagents and microarrays	2.5 h	CE
Greiner/Amplex	GENSPEED MRSA	DE	*S. aureus* (MRSA/LA – MRSA (*mecC*))	Nasal and throat swabs	No	Sampling solution, PCR mastermix, denaturation solution, microarray, localized enzymatic light generation (detected with photodiode arrays)	1 h 15 min	CE

www.greinerbioone.com www.hyplex.de								
Hain Lifescience	23 products based on DNA STRIP technology (line probe assays) and GenoQuick technology	DE	• GenoType MRSA Direct • GenoType Staphylococcus • GenoType MRSA • GenoType Mycobacteria Direct • GenoType MTBC • GenoType M. CM • GenoType M. AS • GenoType MTBDRplus •GenoType MTBDRsl •GenoType LepraeDR •GenoType HelicoDR •GenoType CDiff •GenoType EHEC •GenoType Enterococcus •GenoType Bac-IDent •GenoType BC grampositive •GenoType BC gramnegative •micro-IDent •micro-IDentplus •GenoQuick MTB •GenoQuick MRSA •GenoQuick CT •GenoQuick Bordetella	Patient specimens (blood, urine, swabs, stool, pulmonary, and extrapulmonary samples)	No	Extraction kits for DNA isolation, PCR, DNA STRIPs	GenoType – 4–6 h, GenoQuick – 2.5–3 h	CE

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.hain-lifescience.de								
Fujirebio Europe	INNO-LIPA	BE	HPV genotypes (high risk: 16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82, low-risk: 6, 11, 40, 43, 44, 54, 70, other: 69, 71, 74), HBV genotyping	Cervical scrapes, potential use for biopsies, and paraffin-embedded material	No	Fully automated processing of strips (Auto-LiPA 48) Automated interpretation of the strips is provided with LiRAS® for LiPA HPV (article number: 81085, CE)	3 h	CE
www.fujirebio-europe.com (Innogenetics www.innogenetics.com)								
LG Lifescience	AdvanSure Mycobacteria GenoBlot Assay	KR	25 mycobacteria species	Respiratory samples	No	Extraction buffer, pre-treatment solutions, 2 PCR mixtures, primer mix, strips, buffers, wash solutions, enzyme, and substrate solutions	4 h after DNA isolation	n/a

www.lgls.com LG Lifescience	AdvanSure MDR-TB GenoBlot Assay	KR	*M. tuberculosis*, *rpoB*, *katG*, *inhA*, *ahpC*	Respiratory samples	No	Extraction buffer, pre-treatment solutions, 2 PCR mixtures, primer mix, strips, buffers, wash solutions, enzyme, and substrate solutions	4 h after DNA isolation	n/a
Luminex	xTAG Respiratory Viral Panel (RVP) (xTAG RVPv1/xTAG RVP FAST)	US	Respiratory syncytial virus (RSV), RSV A, RSV B, influenza A, nonspecific influenza A, H1 subtype, H3 subtype, H5 subtype, Influenza B, parainfluenza 1, parainfluenza 2, parainfluenza 3, parainfluenza 4, metapneumovirus (hMPV), adenovirus, entero-rhinovirus, corona NL63, corona HKU1, corona 229E, corona OC43, corona SARS, bocavirus	Nasopharyngeal swabs	No, automated workflow	PCR master mix, reagents for primer extension step, universal tag system, color-coded beads	8 h	IVD

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.luminexcorp.com								
Luminex	xTAG Gastrointestinal Pathogen Panel	US	Bacteria and bacterial toxins (*C.*, *C. difficile*, Toxin A/B, *E. coli* O157, enterotoxigenic *E. coli* (ETEC) LT/ST, Shiga-like Toxin producing *E. coli* (STEC) *stx1/stx2*, *S.*, *S.*, *V. cholerae*, *Y. enterocolitica*), viruses (adenovirus 40/41, norovirus GI/GII, rotavirus A), parasites (*Giardia*, *Cryptosporidium*, *E. histolytica*)	Stool specimens	No, automated workflow	PCR master mix, reagents for primer extension step, universal tag system, color-coded beads	5 h	IVD
Mobidiag	Prove-it Bone and Joint	FI	Gram + (*mecA* meticillin resistance marker, *vanA* vancomycin resistance marker, *vanB* vancomycin resistance marker, *C. perfringens*, *E. casseliflavus*, *E. faecalis*, *E. faecium*,	Synovial fluid, bone biopsy, or tissue samples	No	PCR reagents, Prove-it strip arrays, reagents for HRP catalyzed precipitation reaction	3.5 h (from extracted DNA)	CE

E. gallinarum, *L. monocytogenes*, *S. aureus*, *S. epidermidis*, *S. agalactiae*, *S. dysgalactiae*, subspecies *equisimilis*, *S. pneumoniae*, *S. pyogenes*, Coagulase negative staphylococcus); Gram-(*A. baumannii*, *E. aerogenes*, *E. cloacae*, *E. coli*, *F. necrophorum*, *H. in_uenzae*, *K. kingae*, *K. oxytoca*, *K. pneumoniae*, *M. catarrhalis*, *N. meningitidis*, *P. mirabilis*, *P. vulgaris*, *P. aeruginosa*, *S. enterica* subspecies *enteric*, *S. marcescens*, *S. maltophilia*, *B. fragilis* group, *C. jejuni/coli*, *Enterobacteriaceae*, *N.* sp. non-*meningitidis*)

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
www.mobidiag.com								
Mobidiag	Prove-it Sepsis	FI	*mecA*, *E. faecalis*, *E. faecium*, *L. monocytogenes*, *S. aureus*, *S. agalactiae*, *S. dysgalactiae subspec equisimilis*, *S. epidermidis*, *S. pneumonia*, *S. pyogenes*, *C. perfringens*, *CNS*, *A. baumannii*, *E. aerogenes*, *E. cloacae*, *E. coli*, *H. influenzae*, *K. oxytoca*, *K. pneumoniae*, *N. meningitides*, *P. mirabilis*, *P. aeruginosa*, *S. enterica subs enterica*, *S. marcescens*, *S. maltophilia*, *Bacteroides fragilis group*, *C. jejuni/coli*, *Enterobacteriaceae*	Positive blood culture	No	PCR reagents, Prove-it strip arrays, reagents for HRP catalyzed precipitation reaction	3.5 h	CE
Mobidiag	Prove-it Herpes	FI	*Herpes simplex virus 1*, *herpes simplex virus 2*, *herpes simplex virus 2*, *varicella-zoster virus*, *Epstein-Barr virus*, *cytomegalovirus*, *human herpes virus 6*, *human herpes virus 7*	Cerebral spinal fluid (CSF) samples	No	PCR reagents, Prove-it tube arrays, reagents for HRP catalyzed precipitation reaction	2 h	CE

Nanosphere	Respiratory Virus Plus	US	Influenza A, influenza B, RSV A, RSV B, flu A – 2009 H1N1, flu A – H3, flu A – H1, H275Y (oseltamivir resistance)	Nasopharyngeal swab samples	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of viral RNA via on-board sonication, hybridization to capture probes, washing, hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification	2.5 h	IVD
www.nanosphere.us								
Nanosphere	Gram-Positive Blood Culture	US	Species (*S. aureus*, *S. epidermidis*, *S. lugdunensis*, *S. anginosus* group, *S. agalactiae*, *S. pneumoniae*, *S. pyogenes*, *E. faecalis*, *E. faecium)* genus (*Staphylococcus* spp., *Streptococcus* spp., *M.* spp., *L.* spp.) resistance (*mecA*, *vanA*, *vanB)*	Positive blood culture	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of genomic DNA via on-board sonication, hybridization to capture probes, washing, hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification	2.5 h	IVD

(*continued overleaf*)

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Nanosphere	Gram-Negative Blood Culture	US	Species (*E. coli/S.* spp., *K. pneumoniae*, *K. oxytoca*, *P. aeruginosa*, *S. marcescens)*, genus (*A.* spp., *P.* spp., *C.* spp., *Enterobacter* spp.), resistance (KPC, NDM, CTX-M, VIM, IMP, OXA)	Positive blood culture	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of genomic DNA via on-board sonication, hybridization to capture probes, washing, hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification	2 h	RUO
Nanosphere	*C. difficile*	US	*tcdA*, *tcdB*	Stool samples	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of genomic DNA via on-board sonication, hybridization to capture probes, washing,	2 h	RUO

						hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification		
Nanosphere	Enteric Pathogens	US	Bacteria (*C.* spp., S. spp., *S.* spp., *Vibrio* spp., *Y. enterocolitica*)	Stool samples	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of genomic DNA via on-board sonication, hybridization to capture probes, washing, hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification	2 h	RUO
			Targets, STEC, Shiga toxin gene 1 (*stx1*), Shiga toxin gene 2 (*stx2*), viruses (adenovirus, norovirus, rotavirus)					

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Nanosphere	Respiratory Pathogens	US	Influenza A, flu A – H1, flu A – H3, influenza B, RSV A, RSV B, parainfluenza 1–4, human metapneumovirus, adenovirus, rhinovirus (species A–C)/enterovirus (species A–D), *B. pertussis*, *Bordetella* spp.	Nasopharyngeal swab samples	Yes (extraction tray, pipette tip cartridge, utility tray, test cartridge)	Single use, self-contained cartridge with reagent pack and microarray glass slide for fragmentation of genomic DNA via on-board sonication, hybridization to capture probes, washing, hybridization of gold nanoparticle-labeled detection probes, and silver signal amplification	2 h	RUO
Rheonix	HPV	US	20 different clinically relevant HPV sub-types	Vaginal swabs	Yes (Rheonix CARD Consumable)	PCR reagents, microarray, reagents for HRP catalyzed precipitation reaction	3.5 h	DEV
www.rheonix.com								
Rheonix	Sepsis	US	150 sepsis agents, including C. albicans, *E. coli,* and enterococcus	Whole blood	Yes (Rheonix CARD Consumable)	n/a	3 h	DEV

Rheonix	STI	US	Sexually transmitted infection agents (*N. gonorrhoeae*, *C. trachomatis*, *T. pallidum*, and *T. vaginalis*)	Urine samples	Yes (Rheonix CARD Consumable)	PCR reagents, microarray, reagents for HRP catalyzed precipitation reaction	3.5 h	DEV
Rheonix	HIV	US	HIV screen and confirmatory assay	Saliva samples	Yes (Rheonix CARD Consumable)	n/a	n/a	DEV
Rheonix	UTI	US	Urinary tract infections	Urine samples	Yes (Rheonix CARD Consumable)	n/a	3 h	DEV
Roche	LINEAR ARRAY® HPV Genotyping Test	CH	HPV genotypes 6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, CP6108	Cervical cells collected in PreservCyt® solution	No	PCR reagents, microarray (linear array)	n/a	Canada-IVD CE
Tessarae www.tessarae.com	RPM-Flu	US	>35 different bacterial genera and virus, types of upper respiratory pathogens, incl. influenza, including avian influenza	Throat swabs, nasal washes	No	RPM-Flu 3.1 Kit containing PCR reagents and array, RPM Wash-Stain-Hybridization Kit, additional reagents	10 h	RUO
Tessarae	RPM-TEI	US	>40 different bacterial genera and virus types of tropical and emerging pathogens	Throat swabs, nasal washes	No	RPM-TEI 1.0 Kit containing PCR reagents and array, RPM wash-stain-hybridization kit, additional reagents	10 h	RUO

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Table 4.2 (*Continued*)

Company	Product	CC	Target	Sample	Integration[b)]	Test components	Time to result[c)]	Status[d)]
Tessarae	RPM-HFV	US	Hemorrhagic fever viruses	Throat swabs, nasal washes, blood	No	RPM-HFV 1.0 Array, additional reagents	10 h	RUO
Veredus	VereFlu	SG	Influenza A: H1, H3, H5, H7, H9 with subtype IDs of H1N1, H1N1/2009, H3N2, H5N1 and H9N2, and influenza B	Blood, serum and respiratory swabs (nasopharyngeal, throat, nasal, or throat aspirates)	Partially VereFlu™ chip combines multiplex PCR and Microarray on a single platform	Plastic slide	3 h	IVD
www.vereduslabs.com								
Veredus	VereMTB	SG	10 different mycobacterium strains with special emphasis on *Mycobacterium tuberculosis* complex (MTBC) and its resistance to rifampicin and/or isoniazid	Pulmonary clinical samples, cultured samples	Partially (VereChip)	Plastic slide	3 h	RUO

a) All details are without guarantee.
b) Integration into a sample to answer system.
c) Time to result: Time from adding sample to detection system/assay until result is received.
d) Regulatory status: RUO – research use only; CE – CE-mark (CE-IVD) declaration of conformity EU directive 98/79/EC; DEV – in development; IVD – other regulatory approval for IVD; and n/a – no information found/does not apply.

DNA microarray detection with a comparable LOD of 58 cfu/ml [36]. A DNA microarray for the detection of 10 bacterial pathogens was developed by Kim *et al.* [28]. Here, the microarray detection was combined with multiplex PCR of bacterial specific virulence factors. Results obtained in a validation study with 82 stool samples from patients with foodborne enteritis fully coincided with standard cultivation and PCR approaches. Mao *et al.* [29] developed a DNA microarray for the detection of 14 bacterial pathogens based on universal 16S rDNA primers and species-specific probes [29]. *Shigella* and *Salmonella* could not be identified with this approach because their 16S rDNA sequences were too similar to the *E. coli* sequence. Therefore, additional pathogen-specific probes and primer sets were designed for the detection of *Shigella* and *Salmonella.* This assay was validated with 1500 diarrheal stool samples and 200 samples from healthy individuals and showed a sensitivity of 100% and specificity of 95.2% with an LOD of 10^3 cfu/ml. A magnetic bead-based DNA microarray test for the simultaneous screening of five enteric bacteria and two viruses, which can be read out by the naked eye or a digital camera, was developed by Sun *et al.* [33]. The microarray detection was combined with two sets of asymmetric multiplex PCR systems. The hybridized biotinylated PCR products were detected by streptavidin-coated magnetic beads of diameter 1.05 µm. Tests with 86 samples isolated from patients, fishery products, or the environment revealed both specificity and sensitivity of 100% and an LOD of 50 ng DNA template or 10^3 cfu/ml.

4.3
Antibiotic Resistance Detection

The resistance of widespread Gram-positive and Gram-negative bacteria against antibiotics is a severe and growing issue in healthcare. Infections caused by resistant microorganisms often fail to respond to conventional treatment, resulting in prolonged illness, greater risk of death, and higher costs. A high percentage of hospital-acquired infections are caused by highly resistant bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) or multidrug-resistant Gram-negative bacteria. In the European Union, about 25 000 patients die each year from infections caused by selected multidrug-resistant bacteria and the associated costs are estimated at about 1.5 billion Euros per year [41]. In the United States of America, infections with pathogens resistant to antimicrobials cost the healthcare system in excess of US$ 20 billion annually and generate more than 8 million additional hospital days. The annual societal costs exceed US$ 35 billion [42]. In order to tackle these challenges, a large number of DNA microarray tests have been developed in recent years (Tables 4.3 and 4.4). A few microarrays were developed that cover both Gram-positive and Gram-negative organisms. A broad, high-density microarray, which enabled the identification of 775 genes encoding resistance to aminoglycosides, beta-lactams, chloramphenicols, glycopeptides, heavy metals, lincosamides, macrolides, metronidazoles, polyketides, quaternary ammonium compounds, streptogramins, sulfonamides, tetracyclines,

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Table 4.3 DNA microarrays for antibiotic resistance detection in Gram-positive bacteria.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[45]	ES	Rifampicin- and isoniazid-resistance in *M. tuberculosis*; Chipron LCD Array (Berlin, DE) mutations in *rpoB*, *katG* and *mabA-inhA*-region	46 resistant clinical isolates	Multiplex PCR with biotinylated primer	Hybridization (30 min), enzymatic precipitation reaction (HRP), Chipron transmission scanner	5 h
[46]	UK	47 resistance genes in Gram-negative bacteria	50 *E. coli* from human samples; 37 *S.* from human and veterinary samples	DNA extraction, primer elongation with biotinylated dUTP	Hybridization with 55 probes (20–30-mer), Alere ArrayTube, enzymatic precipitation reaction (HRP/tetramethyl benzidine derivate), Alere ArrayTube Reader (transmission scanner)	n/a
[40]	CA	*A. baumannii*, *P. aeruginosa*, *S. maltophilia*, *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, *H. influenzae*, *L. pneumophila*, *S. aureus*, *S. pneumoniae*, *mecA*, carbapenem resistance genes *(OXA*, *KPC*, *VIM*, *IMP)*, beta-lactam resistance genes *(CTX-M*, *SHV*, *TEM)*	292 reference strains and clinical isolates	Bacterial culture, DNA extraction	Real-time PCR with fluorescence-labeled primer + microarray hybridization in one tube, fluorescence readout after certain cycles	24 h
[47]	CA	189 virulence and 30 antibiotic resistance genes of *E. coli*	3 reference strains, 73 characterized strains from previous studies	Bacterial culture, DNA extraction, no PCR, enzymatic fluorescence labeling (random primer + Klenow-polymerase + Cy5-dCTP), denaturation	Hybridization (overnight) with 348 70-mer probes, fluorescence detection	n/a

[48]	US	12 Gram-positive bacterial gene targets + 3 resistance genes (*mecA*, *vanA*, *vanB*)	1252 positive blood culture samples	Verigene Gram-positive blood culture test (Nanosphere, USA) sample-to-answer system, magnetic bead-based DNA extraction, no PCR	Microarray hybridization, binding of nanoparticle-conjugated detection probes, readout with Verigene Reader	15 h
[49]	IT	Glycopeptide resistance in *Enterococcus*, 42 genes (vanA, vanB, vanC1, vanC2/C3, vanD, vanE, vanG)	13 reference strains, 24 *Enterococci* isolates (human and poultry)	Culture, DNA extraction, no PCR, enzymatic fluorescence labeling (random primer + Klenow-polymerase + Cy5-dCTP), denaturation	Hybridization (1 h) with 105 oligonucleotide probes (approximately 45-mer), fluorescence detection	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis
[44]	US	94 antibiotic resistance genes in Gram-positive and Gram-negative bacteria	51 *E. coli*, *S.*, *Enterococcus* spp., *C. jejuni*	Culture, DNA extraction, no PCR, enzymatic fluorescence labeling (random primer + Klenow-polymerase + Cy5-dCTP)	Hybridization (overnight) with 70-mer probes, fluorescence detection	>24 h
[43]	US	775 antibiotic resistance genes in Gram-positive and Gram-negative bacteria	2 *S. enterica* strains, *MDR S. enterica serovars*, *E. coli*, *C.* spp., *Enterococcus* spp., *methicillin-resistant S. aureus*, *L.* spp., and *C. difficile* reference strains	Culture, DNA extraction, no PCR, enzymatic fluorescence labeling (random primer + Klenow-polymerase + Cy5-dCTP)	Hybridization (overnight) with 70-mer probes, fluorescence detection	>24 h*

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Table 4.3 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[50]	RU	Rifampicin and isoniazid resistance of *M. tuberculosis*; TB-Chip, 27 mutations in *rpoB*, 11 mutations in *katG*, 5 mutations in *inhA*, and mutations in *ahpC*	220 clinical *M. tuberculosis* isolates and 113 sputum samples	DNA extraction, asymmetric PCR with fluorescence-labeled primer	Hybridization (6 h), fluorescence detection with portable reader (Biochip-IMB)	12 h
[51]	JP	Rifampicin, ofloxacin, and dapsone resistance in *M. leprae*, mutations in *rpoB*, *gyrA*, and *folP1*	139 clinical isolates	Multiplex PCR (biotinylated primer), denaturation	Hybridization (1 h), enzymatic precipitation reaction (HRP/TMB), transmission scanner	No clear data: sample prep., 1 h hybridization, washing, HRP reaction, readout, data analysis
[52]	DE	23 resistance genes + 10 toxin genes in *S. aureus*	100 MRSA clinical isolates	DNA extraction, linear multiplex amplification with biotin-labeled dUTP	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	n/a
[53]	DE	185 *S. aureus* genes + 300 alleles thereof including virulence factors and resistance genes	100 clinical isolates and reference strains	DNA extraction, linear multiplex amplification with biotin-labeled dUTP	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	n/a
[54]	DE	*mecA* alleles in *Staphylococci*	78 clincal and veterinary *Staphylococcus* spp.	DNA extraction, linear multiplex amplification with biotin-labeled dUTP	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction, Alere ArrayTube Reader	n/a

[55]	KR	Rifampicin and isoniazid resistance of *M. tuberculosis*; Combichip Mycobacteria (GeneIn Co., Ltd, Busan KR), mutations in *rpoB*, *katG* and *inhA* region	243 clinical isolates (115 RIF resistant strains, 119 isoniazid resistant strains)	Culture, DNA extraction, PCR with biotinylated primer	Hybridization (30 min) on microarray with 25 probes (14–20-mer), streptavidin–Cy5, fluorescence detection	No clear data: sample prep., 0.5 h hybridization, washing, readout, data analysis
[56]	JP	Mutations in seven genes (*inh*, *KatG*, *rpoB*, *rrs*, *rpsL*, *embB)* which are related to *M. tuberculosis* drug-resistance	48 sputum samples from tuberculosis patients	DNA extraction, multiplex PCR	Hybridization (1 h), enzymatic precipitation reaction	No clear data: sample prep., 1 h hybridization, washing, HRP reaction, readout, data analysis
[57]	UK	84 *S. aureus* gene targets including species-specific, antibiotic resistance, toxin, and other virulence-associated genes	43 *S. aureus* isolates	DNA extraction, no PCR	Hybridization with Cy3-labeled gDNA for 16 h with gentle agitation	24 h
[58]	DE	9 *S. aureus* resistance genes (*mecA*, *aacA-aphD*, *tetK*, *tetM*, *vat(A)*, *vat(B)*, *vat(C)*, *erm(A)*, *erm(C)*) and mutation in *grlA*	Reference strains and 13 clinical *S. aureus* isolates	9-plex PCR + *grlA* PCR, labeling by random priming with fluorescence. HighPrime or by photochemical labeling with Psoralen-PEO-Biotin, denaturat.	Hybridization (4 h), fluorescence detection	No clear data: sample prep., 4 h hybridization, washing, readout, data analysis
[59]	CN	8 mutations in the *rpoB* gene of *M. tuberculosis*	102 rifampin-resistance strains	DNA extraction, *rpoB* PCR with fluorescence-labeled primer	Hybridization (1 h), fluorescence detection	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis

(*continued overleaf*)

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Table 4.3 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[60]	CN	6 *Staphylococcal* resistance genes (*mecA*, *blaZ*, *aac*(6′)-*Ie-aph*(2″), *ermA*, *ermC*, *msrA*) und a variable 16S rRNA region for differentiation between *S. aureus* and coagulase-negative *Staphylococcus* (CoNS) species	178 clinical *S. aureus* isolates and 237 CoNS isolates	DNA extraction, multiplex asymmetric PCR (8-plex), denaturation	Hybridization (2 h), fluorescence detection	5 h
[61]	RU	16 and 23 mutations within *gyrA* and *gyrB* gene, respectively (fluoroquinolone resistance), 4 mutations within the *rrs* gene and 5 mutations within the *eis* promoter region (aminoglycoside, and cpreomycin resistance) of *M. tuberculosis*	65 *M. tuberculosis* clinical isolates + 61 clinical specimen (e.g., sputum, bronchioalveolar)	DNA extraction, multiplex PCR with fluorescence-labeled dUTP	Hybridization for 10–16 h, fluorescence detection	24 h
[62]	US	*mecA* (MRSA), SCCmec junction sequence + *S. aureus* specific probe (*tuf*)	87 bacterial isolates + 246 nasal swab samples	DNA extraction, multiplex asymmetric PCR with Cy3-labeled primer to generate single stranded products	Hybridization on Akonni gel element arrays (20–30-mer probes) for 3 h at room temperature without shaking, fluorescence detection	8 h

HRP/TMB: horseradish peroxidase–streptavidin complex/tetramethyl benzidine derivative.

Table 4.4 DNA microarrays for antibiotic resistance detection in Gram-negative bacteria.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[63]	AT	Gram-negative bacteria, 33 genes of beta-lactamases	70 clinical strains	Genomic DNA extraction, amplification of only circular DNA molecules	100-plex assay with padlock probes, microarrays on aldehyde-functionalized glass slides, hybridization (1 h), fluorescence detection	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis
[64]	BE	Gram-negative bacteria, ESBL/AmpC/KPC/NDM-1 assay	207 clinical isolates	DNA extraction, ligation, multiplex PCR	Check-MDR CT101 (Check-Points)	7 h
[65]	DE	*S. enterica*, 77 antimicrobial resistance genes	168 reference strains	Genomic DNA extraction, multiplex linear DNA amplification	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	2 h (after sample prep.)
[66]	UK	Gram-negative bacteria, resistance to 75 clinically relevant antibiotics	132 bacterial isolates	DNA extraction, multiplex linear DNA amplification	Hybridization on Alere ArrayTube platform, enzymatic precipitation reaction (HRP/TMB), Alere ArrayTube Reader (transmission scanner)	5 h (after bacterial culture)
[67]	FR	Gram-negative bacteria, beta-lactamase genes of ESBLs (TEM, SHV, and CTX-M), pAmpC (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR, and CMY-1-like/MOX) and of carbapenemases (KPC, OXA-48, VIM, IMP, NDM)	187 Gram-negative bacilli isolates	Total bacterial DNA extraction, ligation, multiplex PCR	Check-MDR CT103 array (Check-Points)	6.5 h (after DNA isolation)

(*continued overleaf*)

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Table 4.4 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[68]	DE	*A. baumannii*, 91 target sequences associated with 26 antibiotic resistances	Bacterial strains	DNA extraction, three multiplex PCRs	Epoxy-functionalized glass slides, fluorescence detection	4 h (after bacterial culture)
[69]	US	Gram-negative bacteria, important Gram-negative resistance determinants, virulence genes, and replicon markers, *Salmonella* specific markers, and *E. coli* pathogen type markers	*E. coli* and *S. enteric* strains	Total bacterial DNA extraction, biotinylation using a nick-translation kit	Glass slides coated with teflon/epoxy-silane, tyramide signal amplification, hybridization (16–18 h), fluorescence detection	No clear data: sample prep., 16–18 h hybridization, washing, readout, data analysis
[70]	CN	Gram-negative bacteria, SHV genotyping, 54 mutations in the bla_{SHV} gene	1320 clinical isolates	Plasmid DNA extraction, PCR	Microarrays on aldehyde-functionalized glass slides, hybridization (2 h), fluorescence detection	No clear data: sample prep., 2 h hybridization, washing, readout, data analysis
[71]	US	Gram-negative bacteria, clinically important class A beta-lactamase genes and bla_{KPC} gene	102 *Enterobacteriaceae* and 4 *A. baumannii* isolates	Genomic DNA extraction, ligation-mediated amplification	The Check KPC/ESBL (Check-Points)	n/a
[72]	US	*Enterobacteriaceae*, the bla_{ESBL} and bla_{KPC} genes	40 blood samples, 20 non-lactose-fermenting organisms	DNA extraction, ligation-mediated amplification	The Check KPC/ESBL (Check-Points)	18–20 h
[73]	CN	17 categories of drug-resistant genes	*E.coli* reference strains, 42 clinical isolates	Entire bacterial DNA extraction, random amplification, extension with Klenow fragment	Microarrays on aldehyde-functionalized glass slides, hybridization (4 h), fluorescence detection	No clear data: sample prep., 4 h hybridization, washing, readout, data analysis

[74]	US	*Enterobacteriaceae*, the bla_{ESBL} and bla_{KPC} genes	1152 isolates collected from intraabdominal infections	Whole-genomic DNA, ligation-mediated amplification	The Check-KPC ESBL (Check-Points)	n/a
[75]	DE	Gram-negative bacteria, genotyping of bla_{TEM}, bla_{SHV}, bla_{CTX-M} genes	60 clinical isolates from respiratory samples, swabs, urine	Plasmid DNA extraction, PCRs	Epoxy-functionalized glass slides, fluorescence detection	1.5 h (after sample preparation)
[76]	FR	Gram-negative bacteria, most prevalent carbapenemases (NDM, VIM, KPC, OXA-48, and IMP) and extended-spectrum beta-lactamase (ESBL) gene families (SHV, TEM, and CTX-M)	144 Gram-negative strains	Total bacterial DNA extraction, ligation-mediated amplification	Check-MDR CT102 ESBL-Carbapenemase microarray (Check-Points)	6.5 h (after DNA isolation)
[77]	UK	*K. pneumonia*, genotyping of the bla_{KPC} genes	12 reference strains, urine samples	Plasmid DNA extraction, PCR	Epoxy-functionalized glass slides, fluorescence detection ion	5 h
[78]	NL	*Enterobacteriaceae*, the bla_{ESBL} and bla_{KPC} genes	346 clinical isolates	DNA extraction, ligation-mediated amplification	The Check KPC/ESBL (Check-Points)	n/a
[79]	RU	*Enterobacteriaceae*, beta-lactamase genes of class A (TEM, SHV, and CTX-M),	90 clinical isolates	DNA extraction, multiplex PCR	Microarray on porous membranes, biotin as a label, colorimetric detection	4.5 h (after DNA isolation)
[80]	NL	*Enterobacteriaceae*, most prevalent class A ESBL genes	212 *Enterobacteriaceae* isolates	DNA extraction, ligation-mediated amplification	The ESBL Array (Check-Points)	n/a

(*continued overleaf*)

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Table 4.4 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[81]	NL	Gram-negative bacteria, most prevalent carbapenemases (NDM, VIM, KPC, OXA-48, and IMP) and extended-spectrum beta-lactamase (ESBL) gene families (SHV, TEM, and CTX-M)	95 *Enterobacteriaceae* isolates	DNA extraction, ligation-mediated amplification	Check-MDR CT102 ESBL-Carbapenemase microarray (Check-Points)	6.5 h (after DNA isolation)
[82]	RU	*A. baumannii*, *P. aeruginosa*, *Enterobacteriaceae*, 7 types of carbapenemase genes	28 clinical isolates	DNA extraction, multiplex PCR	Microarray on porous membranes, biotin as a label, colorimetric detection	4.5 h (after DNA isolation)
[83]	NL	*Enterobacteriaceae*, the bla_{ESBL} and bla_{KPC} genes	638 *Enterobacteriaceae* isolates	DNA extraction, ligation-mediated amplification	The Check-KPC ESBL (Check-Points)	n/a
[84]	UK	*Enterobacteriaceae*, carbapenemases encoding genes	57 clinical isolates	Total bacterial DNA extraction, ligation of two multi-component probes, PCR	Check-MDR CT102 ESBL-carbapenemase microarray (Check-Points)	6.5 h (after DNA isolation)

and trimethoprims as well as resistance transfer genes of Gram-negative and Gram-positive bacteria, was developed by Frye *et al.* [43]. This microarray covered virtually all antibiotic resistance genes found in the National Centre for Biotechnology Information (NCBI) database, thus reducing the subsequent assays necessary to identify specific resistance gene alleles. This microarray was intended to be used as a screening technique in studies of prevalence, epidemiology, and spread of resistance genes. It was built on an earlier version of a microarray with long 70-mer probes in order to cover all allelic variations of the corresponding resistance gene. This initial version, which was developed by the same group in 2006, covered 94 resistance genes [44].

4.3.1 Gram-Negative Bacteria

The global dissemination of antibiotic resistance in Gram-negative bacteria has received much attention, particularly over the last decades [85]. Transmission of resistance genes among bacteria is highly diverse, involving mutations and horizontal acquisition via plasmids, transposons, and other mobile elements. The *Enterobacteriaceae* are among the most important causes of serious hospital-acquired and community-onset bacterial infections in humans, and resistance to antimicrobial agents in these bacteria has become an increasingly relevant problem [86]. International travel and tourism are important ways for the acquisition and fast spread of antimicrobial-resistant *Enterobacteriaceae* [87]. The variety and heterogeneity of antibiotic resistance markers in Gram-negative bacteria make the development of multiplex systems especially significant. High diversity of certain genetic elements, causing resistance, requires the detection of point mutations together with identification, and this is a feature of resistance in Gram-negative microorganisms. From this standpoint, DNA microarrays are a promising technology, enabling simultaneous detection of hundreds of genetic elements and their point mutations in a single test. It also has the potential for identification of genes playing a role in the transmission of resistance and virulence genes. There are several DNA microarrays described in the literature dealing with the multiplex detection of a large number of resistance genes and virulence factors of Gram-negative bacteria. A microarray developed by Bruant *et al.* [47] for the characterization of *E. coli* isolates contained long 70-mer probes for the detection of 189 virulence factors and 30 resistance genes. The production of bacterial enzymes beta-lactamases was shown to be the most common mechanism of antibiotic resistance in clinically important Gram-negative bacteria [88]. Currently, the term *beta-lactamase* is functional, and the family includes over 900 enzymes, which hydrolyze the beta-lactam ring of the antibiotic. According to the variability in the structure, beta-lactamases are divided into four molecular classes [89]. These enzymes are highly heterogeneous within each class, and certain clinically important types represent the groups of dozens of enzymes, having from one to five point mutations from the parent sequence. Certain mutations can change and expand the substrate activity profile of the enzyme,

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and these enzymes are called *extended-spectrum beta-lactamases* (*ESBLs*). At present, ESBL-producing *Enterobacteriaceae* are the most widespread resistant bacteria worldwide, and they represent a major concern in both hospital and community settings. The last threat in Gram-negative bacteria is associated with the growing distribution of a variety of carbapenemases – beta-lactamases capable of hydrolyzing carbapenem antibiotics – and some of these enzymes were found to be multidrug resistant [90, 91]. Fu *et al.* [73] developed a high-throughput DNA microarray for the detection 17 categories of drug-resistant genes. One-hundred and fifteen oligonucleotide probes were of 42–45 nt length, and they were spotted on aldehyde-functionalized glass slides. Sample preparation involved bacterial DNA extraction, labeling with Cy3-dCTP by a random priming, and extension with Klenow fragment. The $3' \rightarrow 5'$ exonuclease activity of the Klenow fragment ensured the accuracy of the labeled DNA and eliminated the need for primers. The microarrays based on a traditional combination of PCR amplification and fluorescent labeling with following hybridization of glass slides were developed by Dally *et al.* [68] for the determination of 91 resistance determinants in *Acinetobacter baumannii*. The array contained 324 probes for the detection of certain beta-lactamases, aminoglycoside modifying enzymes, efflux systems, 10 resistance-relevant point mutations, and some others. The time for the whole analysis was about 4 h. The method was validated with 60 multidrug-resistant strains, and the results were in complete accordance with the automated susceptibility testing and single PCR results.

The potential of the microarray technology on glass slides with fluorescent detection was successfully demonstrated for the simultaneous determination of the set of point mutations in genes. Leinberger *et al.* [75] developed an integrated microarray for the simultaneous genotyping of the most clinically relevant beta-lactamases of class A (TEM, SHV, and CTX-M types). The microarray included 618 probes for 156 amino acid substitutions, which cover 99% of all TEM variants, 89% of SHV variants, and 50–83% of all CTX-M subgroups, published by the date of study (Figure 4.1). All positions known at that point to be associated with ESBL activity and inhibitor resistance were included in the array. The total assay time was about 5 h after DNA isolation. The sensitivity of ESBL detection was 93%, and genotyping results were 100% concordant with sequencing data. Later, a similar approach was applied for the determination of 54 mutations in SHV type beta-lactamases [70]. The method distinguished 67 variant alleles of the bla_{SHV} gene. It was validated on a collection of 1320 clinical isolates. The results were consistent with the genotypes confirmed by bidirectional sequencing. One of the advantages of microarrays is their ability to distinguish different alleles in one isolate in a single assay. The same technology was successfully applied for the genotyping of KPCs directly from urine specimens [77]. This method combined commercial sample preparation techniques straight from the clinical specimen with allele-specific hybridization on a microarray. The probes were designed to identify single nucleotide polymorphism (SNP) at the four positions of the bla_{KPC} gene. The LOD for target DNA was found to be 10–20 ng per assay depending on target treatment method with DNase and in the 100 cfu/ml range for the SNP

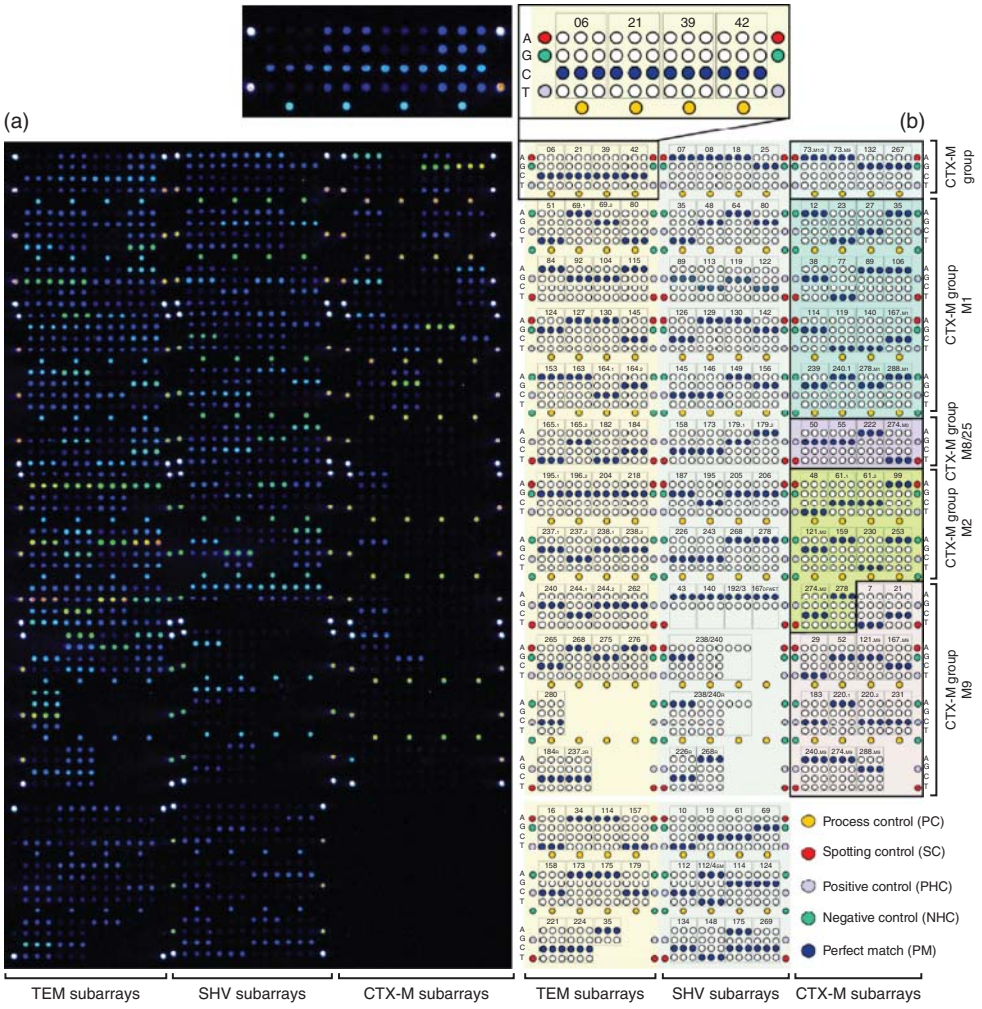

Figure 4.1 DNA microarray for the detection of extended spectrum beta-lactamase genes *bla*TEM, *bla*SHV, or *bla*CTX-M. (a) Fluorescence image after hybridization and (b) oligonucleotide microarray layout [75]. Permissions: http://jcm.asm.org/content/48/2/460.long.

discrimination depending on the target gene and host organism. The reported total assay time was 3.5–5 h after DNA extraction. The need for rapid determination of resistance markers in clinical practice has promotes the development of effective and low-cost identification methods. Therefore, many efforts have been directed toward the improvement of technology and the replacement of its most costly components. A common approach is the replacement of the fluorescent label with biotin, which then is detected with conjugates of streptavidin with enzymes, mostly horseradish peroxidase. The enzyme is detected by an optical method by means of accumulation of a color product on a support. Colorimetric

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microarrays were developed for determining the clinically important ESBLs of TEM, SHV, and CTX-M types in *Enterobacteriaceae* [79] and seven types of carbapenemases in *A. baumannii* and *P. aeruginosa* [82]. The arrays contain several oligonucleotide probes for type and subgroup determination as well as for the detection of important SNPs. Combination of inexpensive membrane supports with inexpensive colorimetric detection makes the method suitable for clinical application. Properties of biotin as a label are attractive because of the small size of its molecule, which has a small effect on the conformation of the target molecule. In addition, the technique of tyramide signal amplification was developed and realized in the amplification kit by Perkin Elmer (Waltham, MA). Biotin as a label, amplified and then coupled with the fluorescent label Alexa 555, was used by Davis *et al.* [69] in the method for the determination of resistance in *E. coli* and *Salmonella enterica*. The array included 203 60-mer oligonucleotide probes, including 117 for resistance genes, 16 for virulence genes, 25 for replicon markers, and 45 other markers. In this work, an important study of inter and intralaboratory validity of the method was carried out. The intralaboratory validity was shown to be high enough in contrast to the interlaboratory reliability. The conclusion was made about the technical complexity of microarray technologies. Each step of the array, including extraction, labeling, hybridization, detection, and data analysis, has a potential for errors and even in the most experienced hands may need troubleshooting to achieve reproducible results. An approach to solve the variability issue lies in reducing the manual process steps and carrying out the complete process in a single tube. It was realized in technology of the ArrayTube by Clondiag, now part of Alere Technologies (Jena, Germany). The ArrayTube platform consists of an array integrated into a micro reaction vial. Labeling by biotin occurs in the course of the multiplex primer extension reaction and is highly specific. The detection is based on colorimetric development of enzymatic reaction. Batchelor *et al.* [46] used the ArrayTube platform for the detection of antibiotic resistance in Gram-negative bacteria. The array contained 55 20–30-mer probes for the identification of genes encoding resistance to aminoglycosides, trimethoprim, sulfonamides, tetracyclines, and beta-lactams, including ESBLs. The assay has been validated in two studies with 50 *E. coli* and 85 *Salmonella* isolates from human and veterinary origin [46, 92]. At present, several arrays for detecting resistance markers are commercially available (see Table 4.2). The AMR-ve Genotyping Kit for the determination of 54 resistance genes in Gram-negative bacteria includes several ESBL markers. The Carbapenemase Typing Kit includes 15 species markers for *Enterobacteriaceae* and 80 genetic markers to identify resistance genes for ESBLs and carbapenemases. Newer developments focused on the ArrayStrip, which combines three individual assays on one microarray. At present, *E. coli* Genotyping is available, which represents a combined assay and includes several hundred markers for the detection of resistance genes, virulence genes, and for H/O serogenotyping. The properties of the ArrayTube and ArrayStrip were recently shown in detecting of Gram-negative bacteria resistant to 75 clinically relevant antibiotics [66] and serogenotyping of *Salmonella* [65]. The method is meant to be easy to use, as all

main steps are performed in a single tube or strip, which aims to decrease manual work and minimize handling errors. The advantage of using microarrays in the strip format is that it is fabricated as a microarray chip at the bottom of each well of a 96-well plate, allowing a large number of genes and up to 96 samples to be tested simultaneously. The same technology platform is used by Check-Points (the Netherlands), which offers the test systems for identification of different beta-lactamase genes: ESBLs, carbapenemases, and AmpCs in various combinations (Check-MDR CT101, 102, 103). The method involves DNA extraction, ligation-mediated PCR, amplification of the ligated probes, and hybridization on the microarray placed at the bottom of the ArrayTube. The hybridization results are developed by colorimetric peroxidase-based detection. Increased attention has recently been focused on the validation of Check-Points commercial systems for the identification of ESBLs and carbapenemases. Eleven publications devoted to the evaluation of Check-MDRs and other kits have been published during last 3 years [64, 67, 72, 74, 76, 78, 80, 81, 83, 84]. This reflects both the special relevance of this area and the prospects of using this technology. Large surveys on Check MDR assays revealed high specificity and sensitivity close to 100%. The method gave good results on most of *Enterobacteriaceae, Pseudomonas* spp., and nonfermenter isolates, but several important for nonfermenter genes were missing. Multiple resistance genes were correctly identified, whatever the complex genetic background of the isolates tested. The method was shown to discriminate effectively between ESBL and non-ESBL beta-lactamases of the same family in one strain, which is difficult to detect with the other methods. At the same time, one of the key mutations of the SHV E240K was reported which was not detected in certain isolates. Some failures in detection of carbapenemases in *K. pneumoniae* isolates and *Enterobacter cloacae* isolate were observed. One of the technique's limitations discussed was that it identified only known genes. Despite some drawbacks, investigators came to the conclusion that these commercially available DNA microarrays presented a practical, rapid and a highly accurate tool for the detection of clinically important beta-lactamase genes. They were recommended as confirmation tests in a routine clinical setting.

The latest development in the field of beta-lactamase gene detection by microarrays was based on a 100-plex assay with padlock probes [63]. The padlock probes with the average nucleotide length of 83 nt were designed for the 33 clinically most common beta-lactamases, including CTX-M, TEM, SHV, KPC, IMP, SME, GES, IMI, PER, RTG, SFO, and VEB. Every padlock probe contains a universal sequence for C2CA (circle-to-circle amplification) and a unique barcode sequence for specific detection on a microarray. The C2CA sequence contained a restriction site, which was necessary for the amplification step. The 5′ and 3′ target recognition arms anneal specifically to a target DNA and are subsequently ligated. These ligated padlock probes are then amplified using C2CA, which consists of two linked rolling cycles. A microarray of 299 oligonucleotides was used for the ligated padlock probe detection. Every sequence-tagged padlock probe was complementary to only one microarray oligonucleotide. The method was shown to provide a very high degree of multiplexing and throughput.

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4.3.2 Gram-Positive Bacteria

The most prevalent causes of bacterial bloodstream infections are Gram-positive bacteria, which account for 52–77% of bacterial sepsis [93]. Bloodstream infections with Gram-positive bacteria are often complicated by antimicrobial resistance. Rapid identification of the bacterial infection and the determination of its antibiotic susceptibility are essential for the correct treatment decision making. The Verigene Gram-Positive Blood Culture test (Nanosphere, Northbrook, USA) enables the identification of 12 Gram-positive bacteria and of three resistance genes (*mecA*, *vanA,* and *vanB*) directly from positive blood cultures (see Table 4.2). The integrated sample-to-answer microarray test has been successfully validated in several multicenter studies with a large number of positive blood cultures [48, 94]. The assay relies on integrated magnetic bead-based DNA extraction and direct target hybridization without PCR amplification, followed by binding of a second nanoparticle-conjugated detection probe. The time-to-result of the test was 2.5 h which was on average 42 h faster compared to the routine culture methods as shown in the validation studies. Another microarray for vancomycin resistance detection was developed by Cassone *et al.* [49]. The microarray contained 105 probes targeting 42 genes within the glycopeptide resistance clusters in *Enterococcus* (*vanA*, *vanB*, *vanC1*, *vanC2/vanC3*, *vanD*, *vanE*, and *vanG*).

4.3.2.1 *Staphylococcus aureus*

MRSA is responsible for a significant proportion of hospital- and community-acquired infections. MRSA strains are resistant to all beta-lactam antibiotics in current clinical use. The resistance is caused by an alternate penicillin-binding protein (PBP2a) which is encoded by the *mecA* gene. There are several *S. aureus* genotyping microarrays [95] which enable the detection of a large number of resistance genes and virulence-associated toxin genes in parallel [52, 53, 57] as well as further DNA microarrays which focus on the detection of *S. aureus* resistance genes [54, 58, 60, 62]. A broad range *S. aureus* genotyping DNA microarray has been designed by Monecke *et al.* [53] based on the ArrayTube platform (see Table 4.2). The microarray allowed the assignment of isolates to strains and clonal complexes in addition to the assessment of virulence and antibiotic resistance. This genotyping microarray has been shown to be a useful tool for rapid strain identification and epidemiological surveillance and outbreak tracing as has been used, for example, to characterize a collection of MRSA isolates from hospitals in the German region of Eastern Saxony [96]. A microarray for the discrimination of *mecA* alleles containing 29 different *mecA* probes was designed by Monecke *et al.* [54] using the Alere ArrayTube platform. A DNA microarray targeting 84 genes targets including species-specific, antibiotic resistance, toxin, and other virulence-associated genes was developed at the University of Nottingham [57]. The microarray allowed the identification of 13 *S. aureus* isolates simultaneously on one chip based on detection of fluorescence-labeled genomic DNA without

prior PCR amplification. A microarray for rapid identification of *S. aureus* antibiotic resistance profiles has been developed by Strommenger *et al.* [58]. The microarray covered 10 clinically relevant antibiotic resistance genes (*mecA*, *aacA-aphD, tetK, tetM, vat*(A), *vat*(B), *vat*(C), *erm*(A), *erm*(C)) including one point mutation in the *grlA* gene and was combined with a previously developed multiplex PCR [97]. Zhu *et al.* developed a DNA microarray for the detection of six antibiotic resistance genes leading to penicillin, methicillin, aminoglycoside, macrolide, lincosamide, and streptogramin B (MLSB) resistance in staphylococci. The array also included probes directed against a variable region in the 16S rRNA gene to discriminate between *S. aureus* and coagulase-negative staphylococci [60].

An example for commercial developments for MRSA detection was developed by Akonni Biosystems (Frederick, USA), which comprises probes directed against the *mecA* gene, the SCC*mec* junction, and the *tuf* gene for *S. aureus* species identification [62]. Three-dimensional gel-based polymer microarrays with integrated probes [98] were combined with multiplex PCR amplification of the extracted genomic DNA. The assay contained seven SCC*mec*-specific primers and three probes to cover different SCC*mec* types and had an LOD of 250 fg genomic DNA, which is equal to approximately 80 cfu. A validation study with 246 nasal swab samples revealed a sensitivity of 80.5% and a specificity of 96.6%. Besides the TruArray MRSA Test, the company's website lists the TruArray MDR-TB Test (*Mycobacterium tuberculosis*, *Mycobacterium avium*, rifampin, and isoniazid resistance) and the TruArray HE Test (*Herpes simplex*, *Varicella zoster*). The Akonni tests are predicated on gel element microarray technology, with increased probe immobilization per unit area compared to conventional planar arrays. This three-dimensional structure is described to both enhance the sensitivity and decrease the hybridization time [99]. The gel element arrays have been shown to have high thermomechanical stability and can be reproducibly manufactured [98]. These attributes translate into sensitive and low-cost consumables and instruments. Akonni's assay development efforts are focused primarily on simplifying the overall microarray workflow for the end user by combining amplification and microarray detection into a single, closed amplicon consumable [100–102]. Example assays demonstrated using this simplified workflow include influenza subtyping with oseltamivir resistance typing, an encephalitis panel of DNA and RNA viruses, and multidrug resistant tuberculosis (TB) [100]. A companion device that Akonni developed for sample preparation is the TruTip, which consists of a binding matrix integrated into a pipette tip [103, 104]. The form factor is both field-portable and amenable to automation. The large porosity of the silica matrix allows easy processing of challenging clinical samples. Simplifying microarray work flow and consumables in this manner leads to lower cost consumables and systems that are better aligned for use at the point of need or in the field (Figure 4.2).

4.3.2.2 Mycobacteria

In 2011, there were an estimated 8.7 million new cases of TB and 1.4 million people died from TB [105]. Multidrug-resistant strains of *Mycobacterium*

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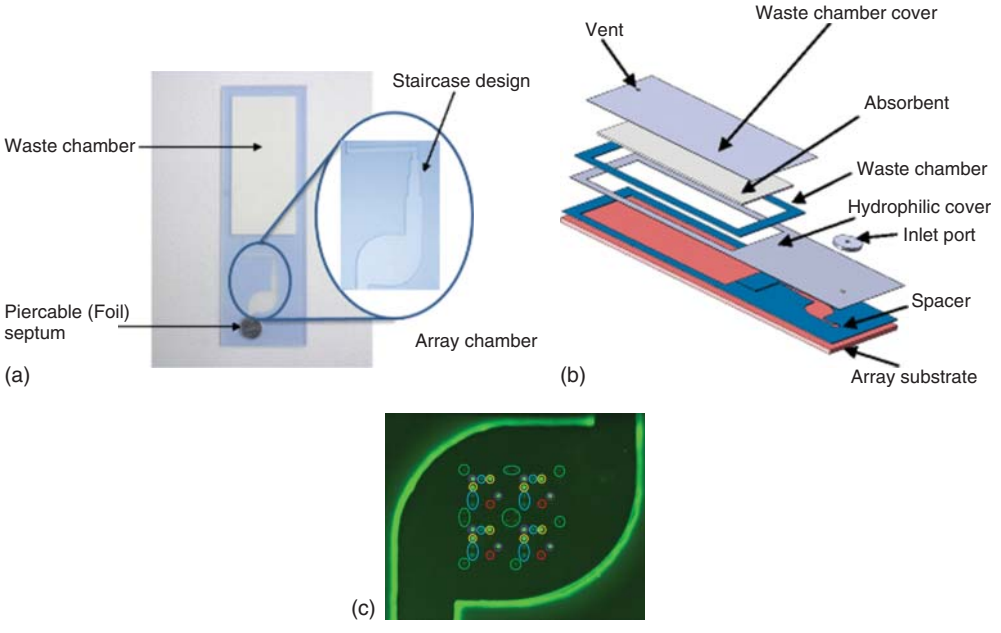

Figure 4.2 Integrated DNA microarray assay. (a) Flow cell, (b) components of the flow cell assembly, and (c) MRSA v4.0 array following on-chip amplification of 105 genomic copies of MRSA [102]. Permissions: http://link.springer.com/article/10.1007%2Fs10544-011-9584-9/fulltext.html.

tuberculosis (MDR-TB) accounted for approximately 5% of the 6.2 million TB cases notified in 2011. Out of these MDR-TB cases, 9% were extensively drug-resistant tuberculosis (XDR-TB), defined as MDR-TB plus resistance to a fluoroquinolone and at least one of three injectable second-line drugs (amikacin, kanamycin, or capreomycin). The development of resistance in *M. tuberculosis* results from mutations in drug resistance genes (*rpoB*, *inhA, katG, pncA, embB, rrs, gyrA,* or *gyrB*). The Engelhardt Institute of Molecular Biology in Moscow (Russia) developed a commercially available microarray test (TB-Biochip) for MDR-TB detection [50], which has been successfully validated in several studies [106, 107]. In 2013, the same group published a new DNA microarray test for XDR-TB diagnosis [61]. Both tests combine multiplex PCR with microarray detection. The XDR-TB assay could detect down to 300 genome copies per assay. The microarray test allowed the diagnosis of XDR-TB within one working day compared to several weeks to months required for traditional culture-based TB drug resistance tests. There are a number of commercially available tests for *M. tuberculosis* available, and they are listed in Table 4.2. Shimizu *et al.* [56] developed a DNA microarray that covered mutations in seven genes (*inh*, *KatG*, *rpoB*, *rrs*, *rpsL*, *embB*) which are responsible for antibiotic resistance against five anti-TB drugs (rifampicin, isoniazid, streptomycin, kanamycin, an ethambutol). The DNA microarray results were compared with standard

culture-based sensitivity testing which took 2 months from sample collection. The DNA microarray showed a high sensitivity (>90%) for all five drug resistances and a high specificity for rifampicin and ethambutol resistance, but a reduced specificity for the detection of isoniazid and kanamycin resistance. A microarray to detect the dominant site mutations at different positions within the *rpoB* gene was developed by Sun *et al.* [59] containing four wild-type and eight mutant probes. Matsuoka *et al.* [51] developed a DNA microarray for the detection of mutations in the *Mycobacterium leprae* genome which confer resistance to key drugs for leprosy treatment. The microarray contained 19 different probes to detect relevant mutations in the *folP1*, *rpoB*, and *gyrA* genes which are responsible for resistance against dapsone, rifampicin, and ofloxacin.

4.4
DNA Microarrays for Virus Diagnostics

During last decades, the field of viral infection diagnostics has developed from the use of immunological tests to wider use of molecular technologies based on genetic identification. DNA chips and microarrays offer effective methods for the rapid detection of genetic variations of viruses and permit a conceptually new approach for viruses testing. The advantages of microarray technology, including a significant increase in productivity and multiplexing, led to the development of methods for the identification of multiple viruses and bacteria in a single assay. The development of a molecular genetic assay for the simultaneous detection of multiple viruses is highly challenging because of the significant genetic diversity even in a single viral species. Viral genomes do not have universal genes such as the 16S or 18S rRNA, which serve as probe targets for bacterial and fungal pathogens. The molecular targets for viruses differ, depending on the conserved genes of each viral group/species. Thus, success in probe design is largely dependent on the amount of sequence data available for the given species. A summary of different DNA-arrays developed during the last 5 years is presented in Table 4.6.

A first microarray designed for detection of a wide range of viruses as pathogens was the ViroChip [108]. The first array version contained 1600 probes derived from the 140 complete viral genomes available in GenBank when the array was designed. The last published version of the array developed contains over 36 000 probes and allow the identification of over 1500 viruses from GenBank submitted by December 2009 [109]. The ViroChip was fabricated by spotting synthesized oligonucleotides on the surface of glass slides. The oligos were 70-mer and matched the sequences common to a taxonomic family, but not found in other families. For some families, the oligos were selected at the genus level. The main advantage of the ViroChip was its ability to identify novel viruses within a known family as the probes were designed against conserved sequences. Different additional probes can be added or deleted if necessary to get the desired spectrum of targets, which is not limited to viruses and may include, for instance, probes for the detection of bacteria and/or fungi. To overcome the limitation

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of spotting tens of thousands of commercially synthesized oligos on the slides, which leads to high cost for each array design, the latest version of the ViroChip utilizes the Agilent ink-jet array platform. Another DNA microarray representing a wide-spectrum approach for pathogen detection is the GreeneChipPm and its part GreeneChipVr [110]. They are high-density oligonucleotide arrays, fabricated using the same Agilent platform as the ViroChip. GreeneChipPm contains a total of about 29 500 60-mer oligonucleotide probes including around 9500 probes for vertebrate viruses together with the probes for pathogenic bacteria, fungi, and protozoa. GreeneChipVr contains the probes for viral infections only. Viral probes were designed to target a minimum of three genomic regions for each family or genus of a virus. In most cases, one highly conserved region was chosen, along with two or more variable regions. Bacterial, fungal, and protozoan probes were selected by a similar strategy, except that the target sequences were chosen only from the 16S ribosomal RNA genes of bacteria and 18S rRNAs of fungi and protozoa. Correct identification of the viruses at the species level was performed when virus-infected cell cultures and clinical samples were tested. Bacterial identification was poorer because of the choice of 16S rRNA as the target gene; and certain bacteria could only be identified at family or class level. The sensitivity of this chip was comparable to that of the ViroChip because of the use of long oligos of the 60-mer.

One of the most comprehensive pathogen detection arrays, which combines identification of bacteria and all known viruses, was designed at the Lawrence Livermore National Laboratory [111]. The Lawrence Livermore Microbial Detection Array (LLMDA) contained target probes for ~900 bacterial and ~2200 viral species. Similar to the GreeneChip, probes from 50 to 66 bases long were selected by two strategies. "Discovery" probes match genome regions that are unique to a taxonomic family or subfamily, but were shared by the species within that family. These probes may also target novel species within a known family. "Census" probes target highly variable regions that are unique to an individual species or strain. They were designed to identify the specific strain of an organism. Bacterial probes were selected from full genome sequences, rather than from 16S rRNA genes as in the GreeneChip, enabling the discrimination at the strain level for most bacteria. The arrays were fabricated by the NimbleGen platform based on photocatalytic synthesis, using a digital micromirror device to control the addition of nucleotides. This technology provides higher probe densities than any spotted oligo arrays or the Agilent inkjet platform, with up to 4.2 million features per array. The advantages of the LLMDA were the broad coverage of bacteria and viruses, as well as of eukaryotic pathogens. Its disadvantages include its cost (compared to spotted arrays such as the ViroChip) and the need (unlike with "universal" arrays) to design new probes periodically to incorporate new genomes deposited in the GenBank. The same array principle was used by Erlandsson *et al.* [112] for the identification of different DNA and RNA viruses. The changes concerned the protocols for sample retreatment and amplification. It was suggested to use multiple displacement amplification (MDA)-based whole genome isothermal amplification by the Phi29 polymerase. The advantage of the method is that both DNA and RNA

viruses could be run through the same protocol. Although subtype identification was not a goal for the probe design, the method discriminated between different virus subtypes. In several samples analyzed, more than one virus was found, including DNA and RNA viruses in some cases. The microarray was proposed for the simultaneous detection of viruses as well as bacteria and could be useful in the diagnosis of diseases with combined viral and bacterial components or those with symptoms that clinically are very similar, making it hard to conclude about the diagnosis. The presented microarray may also be a suitable research tool for finding new pathogens.

Currently, there are certain approaches to the determination of several viruses in a single assay implemented in commercial systems. A resequencing microarray PATHOGENID v2.0 based on technology for high-density oligonucleotide microarrays was developed in collaboration of Affymetrix and the Pasteur Institute. It was designed for the identification of 949 genes, including 126 different viral sequences, 18 of which correspond to highly pathogenic viral agents [113]. The design strategy was based on an alignment covering the whole gene in a search for consensus sequences specific for one gene. The sample preparation involved the following steps: RNA extraction, reverse transcription of cDNA using random primers, and isothermal amplification by Phi29 polymerase. Then the amplicons were fragmented, labeled with fluorescent dyes, and hybridized on the microarrays. The overnight hybridization resulted in relatively long assay time. The method does not require the design of specific primers for all potential targets and the setting up of the corresponding PCRs. This technology was applied for the identification of influenza viruses of different host origins (humans, pigs, horses, and birds) [114], highly pathogenic viruses causing hemorrhagic fever [115], and viruses causing arboviral diseases [116]. The microarray was shown to identify sequences having 13–14% of divergence with the tiled sequence, or stretches of 20 consecutive nucleotides identical to the tiled sequence. The approach allows not only the detection of the currently known isolates but also the discovery of new ones with reliable sequence information.

Another array was developed using technology by Chipron (Berlin, Germany), which sells low-density arrays on transparent, prestructured polymer supports containing eight identical arrays in well-separated, individually addressable hybridization fields. It was used for the determination of the Crimean–Congo hemorrhagic fever virus [117] and the Lassa virus [118]. Colorimetric detection is based on the use of biotin as label of target DNA, which is then developed with a conjugate of streptavidin with horseradish peroxidase. The method is fast and takes less than 1 h after target DNA preparation. It is also inexpensive and simple, and the detection may be performed with the naked eye, and it is proposed for both clinical diagnostic and outbreak investigations (Figure 4.3).

Various commercial vendors are developing or offer DNA microarray-based assays for virus detection (Table 4.2). The technology of Clondiag (Jena, Germany) uses oligonucleotide microarrays on pieces of glass or porous membranes which are placed into a modified standard lab reaction vessel (ArrayTube) (Figure 4.1).

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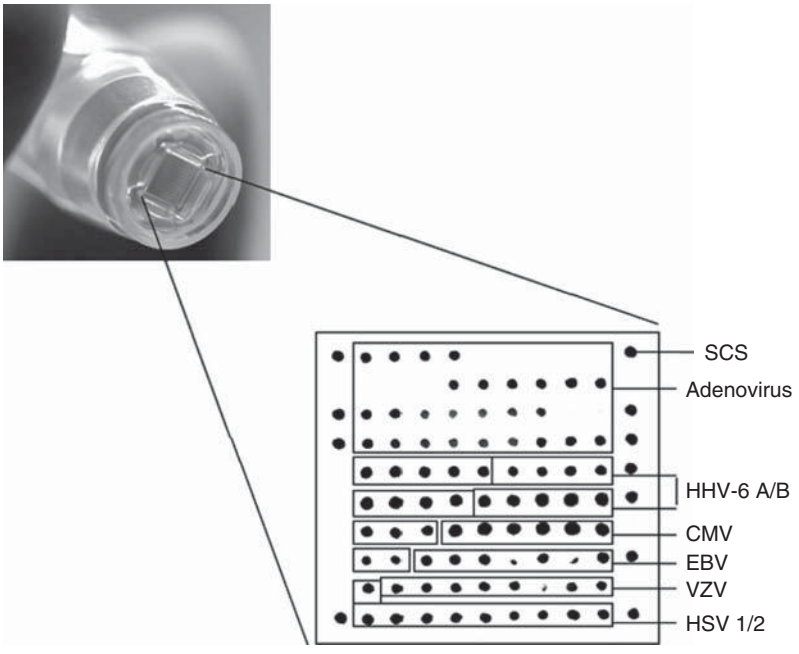

Figure 4.3 VINAray for the detection of human herpesvirus types 1 or 2 (HSV 1/2), varicella zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus-6 types A or B (HHV-6 A/B), and adenovirus genera A–F (adenovirus). The DNA array was based on the ArrayTube platform (Alere, Jena) [119]. Permissions: http://www.journals.elsevier.com/journal-of-virological-methods/.

This makes the performance of hybridization, washing, and detection significantly simpler. Colorimetric detection is based on enzymatic amplification of the signal as in the previous technique. It is performed on a specially designed portable scanner. This technique was applied for the development of VINAray (VIrus DNAmicroarray) to determine herpes simplex virus types 1 and 2, varicella zoster virus (VZV), Epstein–Barr virus (EBV), cytomegalovirus (CMV), and human herpesvirus-6 types A and B [119]. The method was shown to be easy to handle and inexpensive, and allows processing of a large number of samples. The additional advantage of the technique is the compatibility with standard laboratory equipment. Akonni Biosystems (Frederick, USA) developed TruArray HE, a qualitative test for the rapid detection of herpes simplex virus 1, herpes simplex virus 2, and VZV in the cerebrospinal fluid. The test involves workflow-integrated amplification, hybridization, detection, and data analysis. The determination takes 3 h. The TruArray HE is the first array-based test for rapidly detecting and differentiating between three of the most important viral cerebrospinal fluid infections. In the next section, microarrays experimentally developed for identifying individual types of viruses will be described.

4.4.1 Human Papillomaviruses

Human papillomaviruses (HPVs) are causal agents involved in the development of carcinomas in the cervix uteri as well as pathological changes of the skin. Papillomaviruses are small, double-stranded DNA viruses with an icosahedral capsid, and infect the stratified epithelium of mucosal or cutaneous surfaces. The entire HPV class consists of over 120 viruses. The oncogenic activity of HPV is dependent on its genotype. The virus types are classified into high-risk types associated with cervical cancer and recognized as playing a role also in other cancers (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82) and low-risk types known as *causative pathogens of condyloma acuminatum* (types 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and 89) [120]. To ensure correct diagnostics, adequate treatment, monitoring of HPV vaccine implementation, and monitoring of coinfections with multiple HPVs, it is desirable not only to detect HPV but also perform the genotyping assay. Currently, testing and genotyping of HPV is seen as a complement or substitute for analysis of cytology for cervical cancer screening. Various experimental approaches combine the consecutive studies of microarray technique steps: extraction of viral DNA, amplification by multiplex PCR, followed by hybridization on the array. Most established HPV typing assays are based on consensus PCR to amplify the relatively conserved L1 gene region. One of the recently custom-designed microarrays was aimed at the detection of all 120 known HPV genotypes [121]. The microarray was fabricated by Agilent inkjet printing technology for *in situ* synthesis of oligonucleotide probes on a slide. The probe sequences were located on the L1 gene segments and targeted the amplicon sequences of MY of 450 bp and FAP of 480 bp. The final microarray contained in total more than 4300 probes 60-mer in length in six replicates distributed in a random layout. Hybridization of two labeled amplicons took 36 h. Combination of two targeted amplicons improved the assay specificity. It was pointed out that a probable limitation of the array design was lack of target-specific probes for E6/E7 genes. Simultaneous detection of L1, E6, and E7 genes will widen the detection for low- and high-grade dysplasia and cancer. In 2011, Gheit and colleagues demonstrated the APEX (arrayed-primer extension) chip for the genotyping of 19 HPV types [122]. The method involves the hybridization of PCR amplicons with oligonucleotide probes arrayed onto silanized slides. Then oligonucleotides were extended with labeled cyanine 5-ddUTP and detected. The method had been developed previously and repeatedly published [119, 123–125]. With the aim to improve hybridization efficiency and, thus, the analytical performance of the technique, a method with peptide nucleic acids (PNAs) as probes was developed for genotyping of 32 medically important HPVs [126]. PNAs contain uncharged *N*-(2-aminoethyl)-glycine scaffold instead of a negatively charged ribose–phosphate nucleic acid backbone, and nucleotide bases are attached via a methylene carbonyl linker. PNAs can complementarily hybridize with DNA, which makes their application very promising. PNA–DNA duplexes reveal higher stability than their DNA–DNA or DNA–RNA analogs. The synthetic backbone

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of PNAs also means that they are not degraded by nucleases or proteases. This results in a high level of resistance to enzyme degradation and extended lifetime of PNA probes. The total time of PNA arrays starting from DNA extraction, was 4.5 h.

4.4.1.1 Commercial HPV Tests

At present one of the most common tests used to detect genital HPV in cervical samples is the Hybrid Capture II (HCII) (Digene HPV test; Qiagen) assay [127]. The HCII assay is based on hybridization in a solution with a mixture of nonisotopic, single-stranded, full-length RNA probes against 13 high-risk types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68). The assay detects several high-risk types in one reaction and uses a convenient format. However, it does not provide any genotype-specific information. Recently, a new commercially available CE-marked genotyping assay, the CLART HPV 2 (CLART) (Genomica, Madrid, Spain), was developed for the detection and genotyping of 35 HPV types (20 high-risk and 15 low-risk types) in single or multiple infections (Table 4.2). The assay is based on PCR and hybridization on a low-density array. The method was evaluated with 425 cervical samples [128], and the data were compared with the HCII test, which has a defined clinical cutoff. For the detection of 13 high-risk HPV types, both the CLART-13 HR-HPV and the HCII assays showed identical clinical performance, with a clinical sensitivity of 96%. For 17 HR-HPV types, the clinical sensitivity of the CLART assay was 96.9%. The specificity of the CLART and the HCII assays was also very similar. The CLART assay has the advantage in the amount of HPV types, detected in a single test, which is important for the screening and studies required for vaccination trials and for monitoring the efficacy of HPV vaccines.

Another commercially available assay for the detection and genotyping of HPV in routine clinical specimens is CAPH (Clinical Arrays Papillomavirus Humano) (now CLART® HPV2, Table 4.2), Genomica, Spain). In studies, it detected simple or mixed-type infections with 35 HPV types (20 high-risk and 15 low-risk types). The method was validated with 408 clinical samples [129]. A fairly good agreement was found between the results obtained by CAPH assays and HCII. However, in 20.1% of the HCII-positive specimens, the CAPH assay identified LR- and HR-HPV types that were not included in the first assay. These results could point to unspecific cross-reactivity between the probes used for the 13 HR-HPV types included in the HCII kit and other types (i.e., types 6, 11, 53, 61, 66, 70, 72, 81, and 85). False-positive results are less likely in the CAPH assay, since it can detect the 35 most common HPV genotypes. By the CAPH assay, 9.5% of HCII-positive specimens were negative, and 44% of them had a negative Papanicolaou smear test. It was found that screening with HCII tests, followed by HPV detection and genotyping with the CAPH assay, allowed optimal identification of women at high risk for the development of cervical cancer. Furthermore, a better prediction of disease progression was achieved.

The INFINITI HPV-QUAD (AutoGenomics, Carlsbad, CA) assay is a commercially available genotyping platform for the detection and identification of HPV,

in which multiplex PCR is followed by automated processing for allele-specific primer extension and, then, hybridization. The probes target the E1 gene of the HPV genome. The analytical performance of the HPV-QUAD assay was evaluated using liquid cervical cytology specimens, and the results were compared with those obtained using the HCII test (Digene HPV test; Qiagen) [130]. The comparison performed on 197 cervical specimens showed an overall agreement of 83%. Also, it was found possible to use for HPV QUAD different types of specimens including denatured material after the HC2 assay. This may improve the workflow by allowing the testing directly from HC2 assay.

Simultaneously with microarrays on solid supports, high-throughput arrays of another type have been developed. A novel DNA detection assay incorporating the Luminex xMAP suspension array based on using 5.6 mm polystyrene microspheres was applied to the genotyping of HPV in cervical samples [131]. The method was developed for the detection of 26 HPVs (16 high-risk types and 10 low-risk types). To evaluate clinical performance of the liquid bead array, 624 cervical samples were tested, and the HPV genotyping results were compared with those from commercially available systems based on hybridization on solid chips and the DNA sequencing method. The sensitivity for detection of high-risk HPV was 99.0% (liquid bead array), 94.7% (HPV DNA chip (MyGene)), and 85.3% (HCII (Digene Diagnostic)). The suspension array had proven to be as accurate as sequencing in identifying individual HPV types, even in cases with multiple HPV infections. Mistyping between high- and low-risk types was 5.4 and 1.1% for the DNA chip and the liquid bead array methods, respectively. Most of mistyping occurred in samples from multiple infections. The high-level mismatches with DNA chip technology might be explained by the sequence similarity among the 24 probes designed against the 150 bp L1 fragment. As a result, the liquid bead array system demonstrated better performance because of the favorable hybridization kinetics, which was similar to the kinetics of solution-phase hybridization. The accuracy of genotyping in the liquid bead array was comparable to that of the sequencing method. This technique has advantages to be automated or deployed on a high-throughput platform and applied for large-scale epidemiological and vaccination studies.

The use of premanufactured array limits the possibility of identifying new variants of a target or novel pathogen strains. Some approaches use a platform with the ability to synthesize *in situ* the oligonucleotides with unique probe sequences, designed by the researcher. The method of application of the Combimatrix® B3 CustomArray™ technology (Combimatrix, Seattle, USA) for HPV strains detection has been reported [132]. The technology of the CustomArray™ is based on a semiconductor chip bearing up to 95 000 electrodes that can be individually activated digitally, enabling the synthesis of oligonucleotides of different lengths (up to 40 bases) using standard phosphoramidite chemistry. The hybridization of fluorescently labeled DNA takes 16.5 h, which is followed by the fluorescent detection. Different studies were aimed at evaluating the microarray technology useful for the simultaneous detection and typing of HPV in routine clinical diagnostics. From demands of routine clinical requirements, faster methods

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and those that do not need sophisticated equipment and data management are preferable. Several low- to medium-density arrays, based on the hybridization of labeled target DNA with capture probe with fluorescent [133, 134] or colorimetric detection [135, 136], have been developed for HPV genotyping. Another method was based on multiplex amplification of the viral L1 region with improved primers and subsequent ligation reaction of two adjacently hybridized ssDNA probes [137]. The probes constitute a target–specific probe pair, which becomes detectable only if the probes are ligated together in the presence of a complementary target. One of the specific ssDNA probes contained a fluorescent label. After ligation, the mixture is hybridized on the array. Additional ligation step improves the selectivity of the method.

An electrochemical genosensor array method was used for the simultaneous detection of three high-risk human HPVs (16, 18, and 45) found in invasive cervical cancer [138]. The method uses hybridization in a sandwich format on an array consisting of 16 gold working electrodes. Electrochemical detection utilizes horseradish peroxidase as a reporter for second revealing probes. Hybridization time was 1 h. The LODs were 220, 170, and 110 pM for HPV 16, 18, and 45, respectively. To maintain the desired balance of high sensitivity of the assay and the low cost of analysis, many improvements have been made for signal detection and amplification techniques. New developments in HPV testing on microarrays involve the investigation of nanoparticles as labels. A method for introducing luminescent silica nanoparticles into DNA via the classical linkage system of biotin–streptavidin was described by Riccò *et al.* [139]. It was shown that the incorporation of dyes into silica spheres instead of free dyes improves signal intensity and sensitivity of the assay. These nanostructures were found to be suitable labels for the microarrays on glass slides. The principle of light scattering by silica nanoparticles was used to develop a simple DNA chip with visual detection [140]. Silica particles were used as labels for the probes, which, during sandwich hybridization, form a dendrimer-like network of particles which increase markedly the aggregation degree of the particles. The spots with positive hybridization appeared brightly white and visible. Then they may be detected with a flatbed scanner or even by the naked eye. When applied to a target HPV DNA, the LOD was 1 nM by using a flatbed scanner. The total time used for the two-step hybridization analysis, including sample preparation and amplification, was 5–6 h, which is comparable to that of commercial HPV DNA chips with fluorescent detection.

4.4.2 Respiratory Viruses

Detection of acute respiratory infections of viral etiology remains one of the most urgent tasks in clinical virology. In humans, these viruses cause recurrent annual epidemics and have a significant effect on health systems worldwide. Their impact increases when they affect immunocompromised adults or those with chronic deceases, children, and older people. This group of viruses involves respiratory

viruses, influenza viruses, adenoviruses, enteroviruses, and others. The early diagnosis of these viruses is crucial in order to avoid unnecessary antibiotic use, to establish the appropriate treatment, and to prevent infection transmission. Recent advantages in diagnostics have aimed at improving the molecular-genetic methods, their main limitation being caused by genetic variability as many respiratory viruses are RNA viruses. Currently, various commercial molecular assays, which are based on a combination of PCR and hybridization using DNA arrays, have been developed for the identification of respiratory viruses. The Luminex xTAG® Respiratory Viral Panel (RVP) (Luminex Molecular Diagnostics) was the first panel that received FDA approval. It detects influenza A and B, including seasonal H1 and H3, adenovirus, parainfluenza 1–3, respiratory syncytial virus A and B, human metapneumovirus, and human rhinovirus. xTAG RVP showed a sensitivity and specificity of 100 and 91%, respectively, and also had the ability to differentiate human from nonhuman influenza A H1. Its version RVP Fast takes 8–10 h to accomplish the whole procedure [141].

CLART Pneumovir DNA array assay (Genomica, Spain) was developed to detect 11 genera of respiratory viruses simultaneously [142]. This is a low-density microarray with colorimetric detection based on biotin as a label which is introduced in DNA during amplification reaction and is revealed after hybridization with a streptavidin–peroxidase conjugate. The method takes 8 h after nucleic acid extraction and is characterized by high sensitivity and broad detection of respiratory viruses. Its latter version CLART 1 Pneumovir DNA assay allows simultaneous detection and identification of 17 types and subtypes of human respiratory viruses. The results of the array were compared with those of conventional virological methods (direct immunofluorescence and virus isolation) and real-time PCRs for the detection of respiratory viruses [143]. The microarray showed high sensitivity, ranging from 10 to 100 copies of viral genomes per amplification tube, and higher detection rates (91% vs 70%), even for common respiratory viruses (78% vs 68%). The DNA microarray identified 67% of mixed respiratory tract infections, whereas none was detected by the conventional respiratory virus detection assays.

Different microarrays were developed for the subtyping of influenza A viruses. Influenza A virus consists of eight negative single-stranded RNA segments and can be classified into various subtypes based on structure differences of two surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). A total of 16 HA genotypes (H1–H16) and 9 NA genotypes (N1–N9) have been identified. Seasonal epidemics require rapid typing methods for the determination of these alleles. Several microarrays based on classical method of hybridization analysis on glass slides with fluorescent detection were developed, which differed in their approaches for probes design and selection [144–146]. Perhaps the most promising area in applying microarray technology in clinical microbiology is the use of low- or middle-density microarrays. Such arrays may be used for both diagnostic testing and epidemiological investigations. Examples of such arrays for subtyping of influenza A viruses were described by Cannon *et al.* [16] and Heil *et al.* [147]. Several assays were transferred to a microtiterstrip or 96-well plate

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format allowing simultaneous testing of 8–96 samples [148, 149]. In the assays adapted for use in clinical practice, the main improvements concern replacing expensive instrumentation with low-cost detection technology and simplification of the assay performance. Several methods use colorimetric detection based on biotin as a label for target, which is then revealed with streptavidin conjugated to peroxidase [148], a photoinitiator label [150], or gold nanoparticles enhanced by silver staining [151]. The latter method identifies H5N1 viral nucleic acid and simultaneously provides subtype identification of influenza A virus in the absence of target amplification. The target, which is whole viral genome RNA, hybridizes with immobilized capture probes. Then the intermediate oligos with poly A tail form a sandwich complex, which can bind another poly-dT, modified with gold nanoparticles. Twenty samples can be tested in approximately 2.5 h after RNA extraction. A silicon-based microfluidic system was recently developed for the specific detection of viral strains (influenza A and H1N1 2009) [152]. The system was composed of two functional modules: a multiplexed PCR module, and a multiplexed silicon nanowire module for DNA determination. The modules were fabricated by a top-down, standard CMOS technology. The sensitivity achieved was of 20–30 fg μl^{-1} for H1N1 and seasonal FluA in 10 µl of a sample.

4.4.3 Enteric Viruses

An microarray based on the Combimatrix platform was developed for the identification of enteric virus pathogens (norovirus, rotavirus, adenovirus, and astrovirus) [153]. These viruses are important foodborne and waterborne pathogens and frequently cause nonbacterial gastroenteritis worldwide. The probes were of standard types, of discrete tiling types, and of overlapping types. A total of 551 oligonucleotide probes were spotted onto the array. The fluorescently labeled DNA samples were generated by nested PCR. It was demonstrated that the multiplex RT-PCR assay specifically amplified partial sequences of four enteric viruses and the subsequent microarray assay was capable of sensitive and simultaneous detection of those viruses.

4.4.4 Hepatitis Viruses

The hepatitis viruses, named A–E, cause acute and chronic liver disease depending on the virus. Laboratory-based diagnosis usually relies on the detection of specific markers of infection for each virus by the use of immunological tests. Molecular tests for viral nucleic acids began to be used for laboratory diagnostics and in monitoring antiviral treatment efficacy. A DNA microarray on the Affymetrix *in situ* synthesis technology combined with fluorescent detection was developed to identify hepatitis B virus (HBV) genotypes and to detect the mutations in the S, Pol, Core, and X genes [154]. Microarray contained the probes of 20-mer or the detection of 994 mutants located at 298 genomic positions in the HBV

genome. Amplification of whole virus genome makes the assay perspective for the intended clinical use. It allows full analysis of most specimens with more than 5000 genome copies/ml. For samples with lower viral loads, down to approximately 400 genome copies/ml, genotyping and detection of most resistance mutations is still possible. Another microarray for the detection of point mutations in hepatitis B virus associated with antiviral resistance used PNAs to improve the discrimination [155]. The PNA array correctly identified viral mutants and has high concordance (98.3%) with direct sequencing in detecting antiviral-resistant mutations.

A technology of 3D DNA microarrays was applied for the genotyping of hepatitis C virus (HCV) [156]. In this method, oligonucleotide probes are immobilized on the microarray surface in hemispherical hydrogel cells. Immobilization is carried out with the formation of covalent bonds upon ultraviolet irradiation. Assymmetric PCR was used to obtain predominantly single-stranded, fluorescently labeled product. Amplicons were hybridized on the microarray for 14–16 h. The results were 100% concordant for the genotype and 99.7% concordant for the subtype with the results obtained by direct sequencing of the NS5B segment. Several microarrays on the determination of hepatitis viruses used biotin as a label for the target DNA. One of the arrays uses further development with a conjugate of streptavidin with a fluorescent label [157]. This method ensures fast determination because of co-hybridization of target DNA and a conjugate at one stage. In another approach for simultaneous determination of HCV and human immunodeficiency virus type-1 (HIV-1), the development of biotin was performed by a conjugate of streptavidin–gold nanoparticles with following silver staining [158]. In one of the latest developments for the identification of HCV, a single platform was proposed for the determination of virus gene and anti-HCV antibodies [159]. A universal probe of 59 bases was designed, which covered all different HCV genotypes. Thiol-modified oligonucelotides were immobilized onto epoxy-modified glass slides. Fluorescent label Cy 3 was introduced into target DNA in the course of reverse transcription PCR. The efficiency of hybridization at short-time incubations was compared for different reaction formats: direct hybridization of labeled target DNA and indirect hybridization with several labeled detection probes having certain degeneracies for targeting different virus genotypes. Protein probes contained HCV NS4 and core antigen. Serum samples were used as a target, and sheep anti-mouse IgG–Cy3 conjugate developed the immune complexes formed on a protein microarray. Optimization of the assay performance made it possible to reduce significantly the assay time for both antibodies and DNA to approximately 15 min without any loss of sensitivity. This approach is considered a prerequisite for a point-of-care test for the rapid detection of HCV infection in clinical samples.

In recent years, great efforts are being made to develop of miniaturized multiplex microfluidic technology for rapid diagnosis suitable for clinical settings. It intergates the efficient sample handling by microfluidics with the high-throughput parallel analysis on microarrays. A novel microfluidic device with microbead array and quantum dot as labels was developed for HBV genotyping [160]. The integration of a polystyrene beads-based array into microfluidic technology

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combines the rapid binding kinetics of homogeneous assays with improved liquid handling and high sensitivity of the fluorescence detection of quantum dots. The method showed the detection limit of 0.03 nM of synthetic DNA target using TAMRA as label, which corresponded to an absolute detection sensitivity of 1×10^{-16} mol of target molecules in a sample volume of 10 µl. The assay time after sample preparation was less than 1 h. The latest microfluidic microbead-based system for the rapid diagnosis of the HIV-1 has integrated chambers for a sample pretreatment, nucleic acid amplification, cell lysis/hybridization, and an optical detection module [161]. Four primer sets were designed targeting the most conserved regions in vpr, tat, gag, and LTR. The system performed the purification and enrichment of target DNA from infected T cells as well as multiple detection of HIV-1 by the probe-coated beads in real time within 95 min. The microfluidic chips have obvious advantages for fast, accurate, and inexpensive diagnostics. The microarrays recently developed for the identification of some other viruses are listed in Table 4.5.

4.5 DNA Microarrays for Detection of Fungal Pathogens

In recent decades, a clear tendency to increase the frequency and expand the infections caused by opportunistic fungi has been observed worldwide. This occurs as a result of advanced medical treatments and the increase in the number of immunosuppressed patients and patients with autoimmune disorders. These potential pathogens are widely distributed in nature and diverse in their morphological and biological characteristics, the fungi belonging to the genera *Candida, Aspergillus, Rhizopus, Mucor, Absidia, Penicillium*, and so on. They have a common property – the ability for long-term contact with the skin and mucous membranes of humans either without causing lesions or causing them only in the presence of additional factors that inhibit local and systemic host defense mechanisms. Numerous yeasts and molds contribute to clinical diseases, and *Candida* spp. and *Aspergillus* spp. were the most common so far with predominance of *Candida albicans*. But because of changes in treatment strategies, patterns of fungal species have shifted from yeast infections toward a higher proportion of non-albicans *Candida* and molds, including various species of *Aspergillus*, *Fusarium*, and *Mucorales*. An increase in rare mold infections, which are often resistant to antifungal drugs, was also recently observed.

In view of the growing incidence and high mortality rates of invasive mycoses, the effective detection of fungal pathogens in the clinical material and the proper assessment of their value are of great importance both for the individual patients and for understanding the pathology mechanism. Conventional approaches such as microscopy, cultivation, and histopathology are based on morphological and physiological tests, but the diagnosis remains difficult because of limited sensitivity and specificity; also they require several days or even weeks for analysis. Changes in fungal patterns make it necessary to identify a variety of pathogens in

Table 4.5 DNA microarrays for human virus detection.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[109]	US	Over ~1500 viruses, full spectrum of viruses associated with respiratory infections, novel viral pathogens, such as SARS coronavirus and 2009 pandemic H1N1 influenza virus	Nasal swab sample	NA extraction, RT of viral RNA, amplification by random PCR, a smear of 200–1000 bp	Virochip contains ~36 000 probes, fluorescent label Cy3, hybridization (overnight)	~24 h
[110]	US	1710 vertebrate virus species, 135 bacterial, 73 fungal, and 63 parasite genera	Nasopharyngeal aspirates, blood, urine, and tissue	NA extraction, random primed PCR amplification	GreeneChipPm, GreenChipVr, high-density arrays on glass slides, fluorescence detection	No clear data: sample prep., 16 h hybridization, washing, readout, data analysis
[111]	US	Bacteria and all known viruses (including phages), ~2200 virus species, ~900 bacterial species	Fecal, serum, and respiratory samples	RNA extraction. RT to DNA, PCR	Lawrence Livermore Microbial Detection Array (LLMDA), high density oligo microarray, ~38 000 probes for viruses, ~3500 for bacteria, hybridization (16 h), fluorescence label	>24 h
[112]	US	Various viruses and bacteria	Cerebrospinal fluid, urine, serum, cervical smear, feces, skin lesion, and tracheal aspirate	NA extraction, RT of viral RNA, multiple displacement amplification (MDA)-by the Phi29 polymerase	Lawrence Livermore Microbial Detection Array (LLMDA version 2), labeling using nick translation with Cy3-labeled random nanomer primers	>24 h

(*continued overleaf*)

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Table 4.5 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[131]	KR	Human papillomavirus, 26 HPV types (16 high-risk and 10 low-risk types)	624 cervical samples	DNA extraction, Amplification of the L1 gene by PCR	Luminex xMAP suspension array, biotin as a label, development with streptavidin-R-phycoery-thrin, fluorescence detection	n/a
[128]	PT	Human papillomavirus, 35HPV types (20 high-risk and 15 low-risk types)	425 cervical samples	PCR with biotinylated primers, amplification of a fragment a 450 bp fragment within the HPV, L1 region	CLART human papillomavirus 2, low-density microarray, biotin as a label	n/a
[129]	ES	Human papillomavirus, 35 HPV types (20 high risk and 15 low risk)	408 cervical specimens	DNA extraction, multiplex PCR	Microarray hybridization on Genomica HPV test, CAPH (clinical arrays papillomavirus humano)	8 h
[132]	UK	Human papillomavirus	Virus strains	Labeled DNA	Combimatrix B3 CustomArray fluorescent label Cy3 or Cy5, 16.5 h hybridization	No clear data: sample prep., 16.5 h hybridization, washing, readout, data analysis
[121]	US	Human papillomavirus 120 HPV types	Cervicovaginal specimens	Genomic DNA extraction, PCR	Glass slides, fluorescence label Cy3 or Cy 5-dUTP, 36 h hybridization	No clear data: sample prep., 36 h hybridization, washing, readout, data analysis

[138]	ES	Human papillomavirus	Cervical samples	DNA extraction, PCR	Electrochemical genosensor array, microfluidic cell, sandwich format, HRP-labeled secondary reporter probe	No clear data: sample prep., 2 × 20 min hybridization, HRP reaction, readout, data analysis
[139]	IT	Human papillomavirus	DNA strand	PCR	Glass slides, luminescent silica nanoparticles as a label, 3 h hybridization	No clear data: sample prep., 3 h hybridization, washing, readout, data analysis
[140]	KR	Human papillomavirus, synthetic HPV-16 target	The SiHa cell line	DNA extraction, PCR-amplified HPV-16 DNA target	Sandwich DNA hybridization, visual detection based on the light scattering of silica nanoparticles, dendrimer-like silica particle networks	5–6 h
[130]	US	Human papillomavirus	197 cervical specimens	DNA extraction, multiplex PCR, allele specific primer extension	The INFINITI HPV-QUAD assay, film-based chip array, fluorescence label, 1.5 h hybridization	No clear data: sample prep., 1.5 h hybridization, washing, readout, data analysis
[126]	KR	Human papillomavirus, 32 HPV types	72 cervical scrapes	DNA extraction, single-tube nested PCR, one biotinylated primer	Peptide nucleic acid (PNA) probes, Cy5-streptavidin	4.5 h

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Table 4.5 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[134]	KR	Human papillomavirus, 14 high-risk HPV types and 5 low risk types	Cervical specimens	DNA extraction, PCR	HPV 9G DNAChips, fluorescent label Cy 5	1 h (after PCR)
[135]	IT	Human papillomavirus, HPV 6, 16, 31, and 51	—	Real-time PCR	A plastic modular chip, two sequential steps performed on the same device, development with streptavidin–alkaline phosphatase, colorimetric detection	No clear data: 0.5 h hybridization, 0.7 h detection
[133]	DE	Human papillomavirus, 12 HPV types	—	DNA extraction, PCR	36 probes, Streptavidin-Cy5 conjugate, fluorescence detection with CCD camera	3 h
[136]	DE	Human papillomavirus	123 gynecological swab samples	DNA extraction, PCR with biotin labeling, exonuclease digestion	Hydrogel-based biochip assay, Streptavidin-Cy5, Streptavidin-alkaline phosphatase	3 h
[137]	FI	Human papillomavirus, 15 high-risk HPV types and 2 low risk types	Cervical samples	DNA extraction, multiplex PCR, ligation, one of the probes is fluorescence-labeled	Low-density universal microarray	No clear data: sample prep., 2 h hybridization, washing, readout, data analysis
[122]	FR	Human papillomavirus, 19 HPV types	Cervical samples	DNA extraction, multiplex PCR	APEX chip based on primer extension, fluorescence detection (Cy5)	n/a

[142]	FR	11 respiratory viruses, RSV A and B; Flu virus A, B, C; PIV 1, 2, 3, 4A, 4B; hMPV A and B; ADV (33 subtypes), EV (50 subtypes), hRV (65 subtypes); HCoV (subtype 229E); HBoV	Nasopharyngeal or tracheo-bronchic aspirates	RNA extraction, two multiplex RT-PCR or PCR	CLART Pneumovir DNA array (Genomica, Spain), biotin as a label, colorimetric detection with streptavidin–peroxidase conjugate	8 h (after nucleic acid extraction)
[143]	FR	17 respiratory viruses (influenza A including seasonal A/H1N1 and A/H3N2 strains, influenza B, influenza C, parainfluenza 1, 2, 3, 4A, and 4B, hRSV A and B, human rhinoviruses, adenoviruses, EVs specieB, human bocavirus, coronavirus E-229, and the human metapneumovirus A and B	138 nasopharyngeal aspirate samples	DNA/RNA extraction, RT-PCR, or PCR	CLART1 PneumoVir kit V16.3 (Genomica, Spain), biotin as a label, colorimetric detection with streptavidin–peroxidase conjugate	8 h (after nucleic acid extraction)
[114]	FR	Influenza viruses from different host origins (humans, pigs, horses, and birds)	Virus strains	RNA extraction, WTA amplification	PathogenID v2.0 – a resequencing microarray, Affymetrix protocol	n/a
[144]	CN	Influenza A (H1N1) virus	Throat swab specimens	RNA extraction	Aldehyde-activated slides, fluorescence detection (Cy3 or Cy5)	No clear data: sample prep., 2.5 h hybridization, washing, readout, data analysis
[145]	CN	Influenza A virus of five subtypes: H1N1, H1N2, H3N2, H5N1, and H9N2	Virus strains, throat swabs, and nasopharyngeal swabs	RNA extraction, RT-PCR	Aldehyde-modified glass fluorescent label Cy3	7 h

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Table 4.5 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[146]	RU	Influenza A virus	Tissue culture	Genomic RNA isolation, RT-PCR	Fluorescence detection	No clear data: sample prep., 2 h hybridization, washing, readout, data analysis
[150]	US	Influenza	—	RNA extraction, RT-PCR, lambda exonuclease treatment	The ampliPHOX colorimetric detection technology	6 h
[151]	US	Major influenza A virus strains (H1N1, H3N2)	Virus isolates	RNA extraction, PCR-free	Hybridization in sandwich format, gold nanoparticle as a label, silver staining	2.5 h (after RNA extraction) for 20 samples
[16]	IE	16 viral and two atypical bacterial respiratory pathogens	Throat swabs	NA extraction, PCR	Low density oligonucleotide microarray, biotin as a label, development with streptavidin–horseradish peroxidize (15 min)	No clear data: sample prep., 1 h hybridization, washing, HRP reaction (15 min), readout, data analysis
[147]	US	Influenza A/H1N1 of various species origin (human H1N1, human H3N2, 2009 pandemic influenza A H1N1, swine H1N1, and a wide variety of avian, equine, canine, and swine influenza viruses)	—	RNA extraction, RT-PCR	MChip, a low density microarray, artificial neural network (ANN)	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis

[148]	DE	Influenza virus A	Throat swabs, bronchoalveolar lavage	RNA extraction, RT PCR	Microarray in 8-well microstrip format, Biotin as a label, colorimetric detection based on horseradish peroxidase	No clear data: sample prep., 1 h hybridization, washing, HRP reaction, readout, data analysis
[149]	FI	10 common adenovirus genotypes causing human infections	Nasopharyngeal specimens	RT-PCR	Microarrays in 96-well microtiter plates, photoluminescence	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis
[152]	SG	Influenza A (H1N1)	DNA templates from hemagglutinin gene and polymerase basic protein 2	PCR	Silicon-based microfluidic system	No clear data: PCR, hybridization (0.25–0.33 h)
[162]	FI	Enteroviruses (*Picornaviridae* family)	Virus cell cultures	RNA extraction, RT-PCR, Cy5-labeled reverse primer	Microarrays in 96-well microtiter plates	No clear data: sample prep., 1 h hybridization, washing, readout, data analysis
[153]	KR	Enteroviruses: Adenoviruses (AdVs), astroviruses (AstVs), noroviruses (NoVs), and rotaviruses (RoVs)	Patients' stool samples	NA extraction, multiplex RT-PCR	The CombiMatrix CustomArray (Macrogen, KR), fluorescent label Cy3	19–20 h

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Table 4.5 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[159]	UK	Hepatitis C virus	Serum	Total RNA extraction, RT-PCR	DNA microarray test to detect HCV RNA and a protein microarray to detect human anti-HCV antibodies on a single platform, fluorescence detection (Cy3)	0.25 h (after RT-PCR)
[156]	FR	Hepatitis C virus	345 positive serum samples	RNA extraction, asymmetric PCR	Low-density hydrogel-based biochips, fluorescence detection	No clear data: sample prep., 14–16 h hybridization, washing, readout, data analysis
[154]	FR	Hepatitis B virus genotypes and mutations	Serum	DNA extraction	The Affymetrix in situ synthesis technology, fluorescent label	n/a
[155]	KR	Hepatitis B virus, antiviral resistance	68 Serum samples	DNA extraction, the single-closed-tube nested PCR method	An array using peptide nucleic acid (PNA) biotin-labeled PCR fragments Cy5–streptavidin	n/a
[157]	KR	Hepatitis C virus	Serum	RT-assym PCR	Biotin-labeled PCR product and streptavidin–Cy3 conjugate were cohybridized simultaneously	<2 h (after amplification)

[160]	CN	Hepatitis B virus	HBV DNA samples	PCR, T7 *in vitro* transcription	Microfluidic chip, SuperAvidinTM coated polystyrene beads, quantum dot as labels	<1 h (after sample prep.)
[163]	CN	Hepatitis C virus	607 positive serum samples	RNA extraction	An oligonucleotide chip genotyping test, colorimetric detection	n/a
[158]	CN	Human immunodeficiency virus type-1 (HIV-1) and hepatitis C virus	Blood	NA extraction, Multiplex asymmetric PCR	Glass slides, biotin as a label, gold label silver staining, visual detection	No clear data: sample prep., 3 h hybridization, washing, silver staining
[161]	TW	Human immunodeficiency virus-1 (HIV-1)	HIV-1 cell line samples	—	Microfluidic system using magnetic beads, optical detection	2 h
[164]	FR	9 DNA and RNA neurotropic viruses: herpes simplex virus 1 (HSV-1), HSV-2, varicella-zoster virus (VZV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), HHV-7, HHV-8, and the human enteroviruses (HEVs)	Cerebrospinal fluid	Total NA extraction, Viral genome-specific fragment amplification by multiplex PCR	Arrays in eight-well strips, colorimetric detection	n/a
[119]	DE	Herpes simplex virus types 1 and 2, varicella zoster virus, cytomegalovirus, Epstein–Barr virus, human herpesvirus-6 types A and B, and adenovirus genera A–F	Plasma	DNA extraction, PCR	VINAray, CLONDIAG chip Technologies, ArrayTube, biotin as a label, horseradish peroxidase–streptavidin conjugate	No clear data: sample prep., 1 h hybridization, washing, HRP reaction, readout, data analysis

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Table 4.5 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[115]	FR	Highly pathogenic viruses Several hemorrhagic fevers Crimean–Congo hemorrhagic fever virus, five different families of BSL-4 viruses (*Arenaviridae*, *Bunyaviridae*, *Filoviridae*, *Flaviviridae*, and *Paramyxoviridae*)	Infected cell supernatant, organ, serum	RNA extraction, reverse transcription, Isothermal amplification by Phi29 polymerase	High-density resequencing microarray PATHOGENID v2.0	48 h
[117]	DE	Crimean-Congo hemorrhagic fever virus	63 serum samples	RNA extraction, RT-PCR	Low-density macroarrays on polymer support, biotin as a label, colorimetric detection	No clear data: sample prep., 0.5 h hybridization, washing, readout, data analysis
[118]	DE	Lassa Virus	Serum	RNA extraction, RT PCR	Custom-made array by Chipron, colorimetric detection based on horseradish peroxidase	n/a

Table 4.6 DNA microarrays for human fungal pathogen detection.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[165]	TW	20 species of fungi *Acrophialophora Fusispora Aspergillus* spp., *Candida* spp., *Paecilomyces*, and other	75 sputum samples	DNA extraction, PCR amplification of ITS regions with universal primers	Middle density arrays on nylon membrane, digoxigenin as a label, development with antidigoxigenin antibodies-alkaline phosphatase, colorimetric detection	8 h (after DNA extraction)
[166]	IT	24 species belonging to 10 genera, *Candida* spp. *Aspergillus* spp., *Trichophyton species*, *C. neoformans*, *T. cutaneum*, *E. floccosum*, *F. solani*, *M. canis*, *P. marneffei*, and *S. cerevisiae*	20 clinical fungal isolates	DNA extraction, amplification of the regions of ITS1, ITS2, and the 5.8S rRNA gene with universal primers	Microarray based on arrayed-primer extension technique (APEX), fluorescence detection (Cy5)	5–6 h (after DNA extraction)
[167]	TW	64 fungal species	57 clinical isolates	DNA extraction, amplification of the ITS regions and a part of the 5.8S ribosomal DNA with universal primers	Microarrays on a nylon membrane, digoxigenin as a label, development with antidigoxigenin antibodies-alkaline phosphatase, colorimetric detection	24 h (starting from isolated colonies)
[168]	TW	77 yeast species (16 genera) and 66 mold species (33 genera) *Candida* spp. *Aspergillus* spp. *Trichosporon*	116 blood samples	DNA extraction, amplification of the ITS regions with universal primers	Two middle density arrays on nylon membrane, digoxigenin as a label, development with antidigoxigenin antibodies-alkaline phosphatase, colorimetric detection	16–24 h (from the time at which growth was detected)

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Table 4.6 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[169]	DE	12 target species *Candida* spp. *Aspergillus* spp.	21 strains from clinical isolates	DNA extraction, amplification of the ITS regions with universal primers	Oligonucleotide microarray on glass slide, fluorescence detection (Cy3), data normalization	4 h (after DNA extraction)
[170]	ZA	Potentially mycotoxigenic fungi:a total of 40 food-borne fungi including *Alternaria*, *Aspergillus*, *Bipolaris*, *Claviceps*, *Curvularia*, *Diplodia*, *Drechslera*, *Eurotium*, *Fusarium*, *Penicillium*, and *Pithomyces*	Fungal strains	Total genomic fungal DNA extraction, labeling without amplification	Microarrays on glass slides, fluorescence detection (Cy3 or Cy5)	No clear data: sample prep., 24 h hybridization, washing, readout, data analysis
[171]	TW	23 medically important mold pathogens including *Aspergillus* spp.,*Fusarium* spp., *Mucor* spp.,*Rhizopus* spp., *R. pusillus*,*P. marneffei*, *S. vasiformis*, *A. elegans*, *L. corymbifer*, and *S. racemosum*	Blood samples	DNA extraction, PCR with universal primers	Microsphere-based suspension array, biotin as a label, development with streptavidin-R-phycoerythrin, fluorescence detection	5 h
[110]	US	1710 vertebrate virus species, 135 bacterial, 73 fungal, and 63 parasite genera	Nasopharyngeal aspirates, blood, urine, and tissue	NA extraction, random primed PCR amplification	GreeneChipPm, high-density array on glass slides, fluorescence detection	No clear data: sample prep., 16 h hybridization, washing, readout, data analysis

[172]	JP	26 fungal species *Candida* spp. *Aspergillus* spp. *Trichosporon* spp.	Nail specimens, fungal strains	DNA extraction, amplification of full ITS sequence	Microarrays on glass slides fabricated by Bubble Jet technology, fluorescence detection (Cy3)	No clear data: sample prep., 4 h hybridization, washing, readout, data analysis
[173]	DE	14 fungal pathogens (*Aspergillus* spp. *Candida* spp., *F. oxysporum*, *F. solani*, *M. racemosus*, *R. microsporus*, *S. prolificans*, and *T. asahii*)	Blood, bronchoalveolar lavage, and tissue samples	DNA extraction, multiplex PCR for amplification parts of 18S, 5.8S, and ITS 1 regions of the fungal rRNA genes	Oligonucleotide microarray on glass slide, fluorescence detection (Cy3)	n/a
[35]	KR	50 bacteria and 7 pathogens causing bloodstream infections (*Candida* spp., *Aspergillus* spp., *Cryptococcus* spp.)	Blood	Genomic DNA extraction, Two PCRs with universal primers	Glass slides, fluorescence detection	10 h (from a positive blood culture)

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one test. The necessity of fast methods thus becomes more and more apparent. Molecular-based techniques are becoming attractive because of their sensitivity and specificity, as well as the arrays due to their multiplexing properties and reducing the analysis time. In the past few years, DNA array technology has been used to identify a variety of yeasts and molds. A summary of various arrays developed is presented in Table 4.6. In most cases, oligonucleotide capture probes were used for targeting sequences of the ITS regions of the rRNA gene cassette. A high-density microarray GreeneChipPm designed for detection of a wide range of pathogens involving bacteria, viral, parasite, and 73 fungal genera was developed on the Agilent platform [110]. This DNA microarray allowed the detection of the microbial pathogens causing bloodstream infections and clinically dominant fungi such as *Candida*, *Aspergillus*, and *Cryptococcus*, which account for more than 10% of the pathogens in septicemia. The identification was based on oligonucleotide probes of 60-mer, which were synthesized on glass slides by using an inkjet deposition system. The probes were targeted at the 18S rRNA gene. Another high-density microarray was developed for the identification of 50 bacteria and 7 fungi in blood [35]. As *C. albicans* is an important fungus responsible for more than 50% of fungal bloodstream infections and is associated with high mortality, it was identified at the genus level by selecting a highly conserved regions. *Candida tropicalis* and *Candida parapsilosis*, *Aspergillus*, and *Cryptococcus* were also detected at the genus level. *Candida glabrata* and *Candida krusei* were identified at the species level because of their resistance to fluconazole. The probes were designed on the basis of the ITS regions of the 18S rRNA. Together with the genus- and species-specific probes, the array includes two broad-ranged fungi probes to reduce amount of false-negative results which might be obtained as a result of the sequence diversity among clinical species.

The middle-density microarray on glass slides with fluorescent detection was developed in 2005 [169]. Capture probes of 16–24 bases were complementary to the ITS1 and ITS2 regions of the fungal rRNA genes. The method allowed the discrimination between 12 most common pathogenic *Candida* and *Aspergillus* organisms at the species level. It was the first microarray with a special quantification technique by a normalization procedure to enhance the specificity of the results. DNA microarray based on the same technique was also developed for the determination of for the analysis of neutropenic patients with invasive fungal infections [173]. It uses longer capture probes of 22–70 bases, which were targeted for the 18S, 5.8S, and ITS 1 regions of the fungal rRNA genes. Although universal fungal primers are widely used for target amplification, the authors designed the primers for amplification of the ITS1 region to improve the hybridization efficiency by reducing the amplicon size. It allowed the investigation of noncultured clinical samples. Another array on glass slides with fluorescent detection was constructed for the identification of 26 fungal species [172]. Full ITS regions including the 5.8S rRNA gene were chosen as target nucleic acid to improve the identification at the species level. They were amplified by PCR with universal primers. Capture probes of 20–29 bases were designed for discrimination both at the genus and species level. This type of array was

fabricated using Bubble Jet technology, in which thiolated oligonucleotides were immobilized onto a glass surface modified with maleimide groups. It was found that there was a possibility for false-positive hybridization results for specimens from healthy controls. The reason was related to commensal fungi, which are unavoidable in the clinical setting. Hence, a positive result of fungal DNA by microarrays is the rationale for a more detailed diagnostics.

Further improvements in microarray technology allowed the development of an identification method for oligonucleotide microarray for the identification of potential mycotoxigenic fungi without amplification of target DNA [170]. It was fluorescently labeled using a random approach. This labeling strategy was applied to reduce the secondary structures of DNA. The probes of 14–25 bases were complementary to the sequence variations in the elongation factor 1-alpha and the ITS regions of the rRNA gene. The array contains different probes – genus and species-specific probes and those specific for genes leading to toxin production. A combination of multiple probes for each species improves the discriminatory power of the assay because of the decrease in cross-hybridization, which may be a problem in the identification of different fungal species. Another approach consisted in the introduction of LNAs (locked nucleic acids) into the probe structure. It was also shown to improve the hybridization stringency and decrease cross-hybridization. Microarrays on porous membrane supports with alternative colorimetric detection were developed for the determination of 64 clinically relevant molds [167]. The capture probes were designed against the ITS1 and ITS2 regions of the fungal rRNA genes of filamentous fungi. The detection procedure uses digoxigenin as a label for the target DNA. It is revealed with a conjugate of antidigoxigenin antibodies with alkaline phosphatase. The enzyme catalyzes then the formation of a colored product adsorbed on a surface of the membrane. Later, the same method was adopted for the determination of 20 fungal species in the sputum of patients with cystic fibrosis [165]. It was shown that the technique was relatively simple, time-saving, and more sensitive than determination in a culture. However, neither the array method nor cultures allow differentiation between colonization and a true respiratory infection. At the same time, early detection of colonization is of importance for the initiation of an appropriate antifungal therapy in order to eradicate it before chronic colonization can develop. The presence of fungal DNA was detected in several samples which were negative in culture, which was then confirmed cloning and resequencing of the ITS fragments. A similar colorimetric DNA array was developed for the identification of bloodstream fungal infections [168]. The method involves two arrays and allows identifying 77 yeast species and 66 mold species.

An oligonucleotide microarray based on the APEX technique was developed for the identification of 24 fungal species belonging to 10 genera frequently isolated from invasive and superficial infections [166]. Species-specific oligonucleotide probes were complementary to the ITS1 and ITS2 regions. The labeling of PCR products was not required. The method was based on complementary hybridization of target DNA with immobilized probes and specific extension of a single base at the 3′ side of the anchored probe. Negligible level of cross-hybridization

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was detected. The feature of the array was its excellent discrimination power even for highly related species such as *C. parapsilosis*, *Candida orthopsilosis*, and *Candida metapsilosis*. One of the newest developments is a microsphere-based suspension array for the identification of 23 clinically relevant filamentous molds and dimorphic fungi [171]. The group- and species-specific probes were targeted at the ITS2 region. The probes were coupled to carboxylated beads. After hybridization with target DNA labeled by biotin, the beads were incubated with a conjugate of streptavidin-R-phycoerythrin, and then the fluorescence was analyzed by a special suspension array system. The method revealed high specificity, as all species- and groups-specific probes were correctly targeted to the strains. The achieved sensitivity showed that the method could be applied for the detection of fungal species in human blood.

4.6 DNA Microarrays for Parasite Diagnostics

4.6.1 Malaria

Parasite infections are among the most burdensome diseases for humans, especially in low-income developing countries. Malaria, caused by the *Plasmodium falciparum*, infects millions of people worldwide and is responsible for over a million deaths worldwide per year, and the treatment options are limited to a few antimalarial drugs such as sulfadoxine–pyrimethamine [174]. Unfortunately, antimalarial drug resistance is on the rise. Drug resistance is often caused by gene duplication or SNPs. The latter genetic markers are highly suitable targets for DNA microarray analysis, and thus several studies were conducted and published using DNA microarray-based SNP tying for the detection of antimalarial drug resistance. Andreas Crameri published a study, as part of the ResMalChip FP5 project and supported by further funders, on the development and application of a DNA microarray for the genotyping of 34 SNPs in five genes, that is, *pfdhfr*, *pfdhps*, *pfcrt*, *pfmdr1*, and *pfATPase* [175]. The assay comprised a PCR amplification of the target regions, followed by a nested PCR approach, two color primer extension, and DNA microarray hybridization. For the full four-base SNP typing, two separate reactions were required.

The authors rated the genotyping assay to be highly suitable for low-resource settings and calculated the assay costs of 0.27 euro per SNP and an assay time of 15 h for four 96-well plates. The limit of detection given was 10–100 parasites. They also described the assay as suitable to cope with the problem of multiplicity of infections and the determination of the most dominant haplotype representing the clinical malaria-causing clone. While the present study only worked with cultured organisms, the authors and collaborators conducted further successful field studies on the basis of the microarray system using patient samples in Tanzania [176], Niger [177], Papua New Guinea [178], and the Solomon Islands [179]. In an interesting study, the group around Hans-Peter Beck used the same

technological approach to genotype polymorphisms, that is, *CYP2A6*, *CYP2B6*, *CYP2C8*, *CYP2C9*, *CYP2C19*, *CYP2D6*, *CYP3A4*, *CYP3A5*, and *NAT2*, which are known to have an impact on human malarial drug metabolisms [180]. The authors concluded that microarrays were not the ideal tool for this purpose due to a lower robustness in their study and projected a stronger impact of sequencing for future epidemiological studies.

In a related publication, Steenkeste *et al.* [181] describe further development of the previous primer extension/microarray into a zip-code array, called *FlexiChip*. The system, which combined the four color primer extension reaction with a zip code array, was designed using a previously described protocol [182] to overcome the target limitation by the previous microarray assay [175] (Figure 4.4).

The monitoring of drug resistance SNPs in *Plasmodium falciparum* was also approached using the Luminex Xmap technology by creating liquid-phase arrays and ligase reactions for SNP genotyping [183, 184]. Comparative genome hybridization for was done using Affymetrix scrMalaria arrays (custom 25-mer oligonucleotide arrays, 5159 predicted genes, 260 596 probes) [185], spotted 70-mer DNA microarrays [186], and microarrays based on the Nimble Gen platform [187]. Genomic mapping technologies were reviewed by Su *et al.* [188]. While of high scientific value for the study of genome variation in *P. falciparum*, these studies have no direct relevance for diagnostic microbiology. In a study led by the Chinese Centre for Disease Control and Prevention [189], a DNA oligonucleotide microarray was designed to detect SNPs in the five resistance-conferring genes (chloroquine resistance transporter (*Pfcrt*), multidrug resistance 1 (*Pfmdr1*), dihydrofolate reductase (*Pfdhfr*), dihydropteroate synthetase (*Pfdhps*), ATPase 6 (*PfATPase6*) in *P. falciparum* in combination with a multiplex PCR preanalytical regimen. Cross-reactivity studies were successfully performed using genomic DNA isolated from *Plasmodium berghei*, *Plasmodium cynomolgi,* and *Plasmodium vivax.* As not all example isolates for all potential resistance genotype could be obtained, a series of 20 combinations of 37 synthetic oligonucleotides (50-mer) were tested. The array assay showed good performance and was considered a promising tool for resistance-conferring SNP detection in *P. falciparum.* It is not known whether the system was used in subsequent studies, as no related citing articles were found.

4.6.2
Waterborne Parasites

Water supply is a major factor in managing risk by microbial contamination and human parasites. The traditional approach by which water quality is assessed, that is, by monitoring indicator organisms such as coliform bacteria derived from the human fecal flora, is increasingly considered insufficient and being replaced by the detection of specific pathogens such as *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* as leading examples for human eukaryotic parasites. Consequently, a number of studies focused on the molecular detection of these organisms, and DNA microarray-based publications

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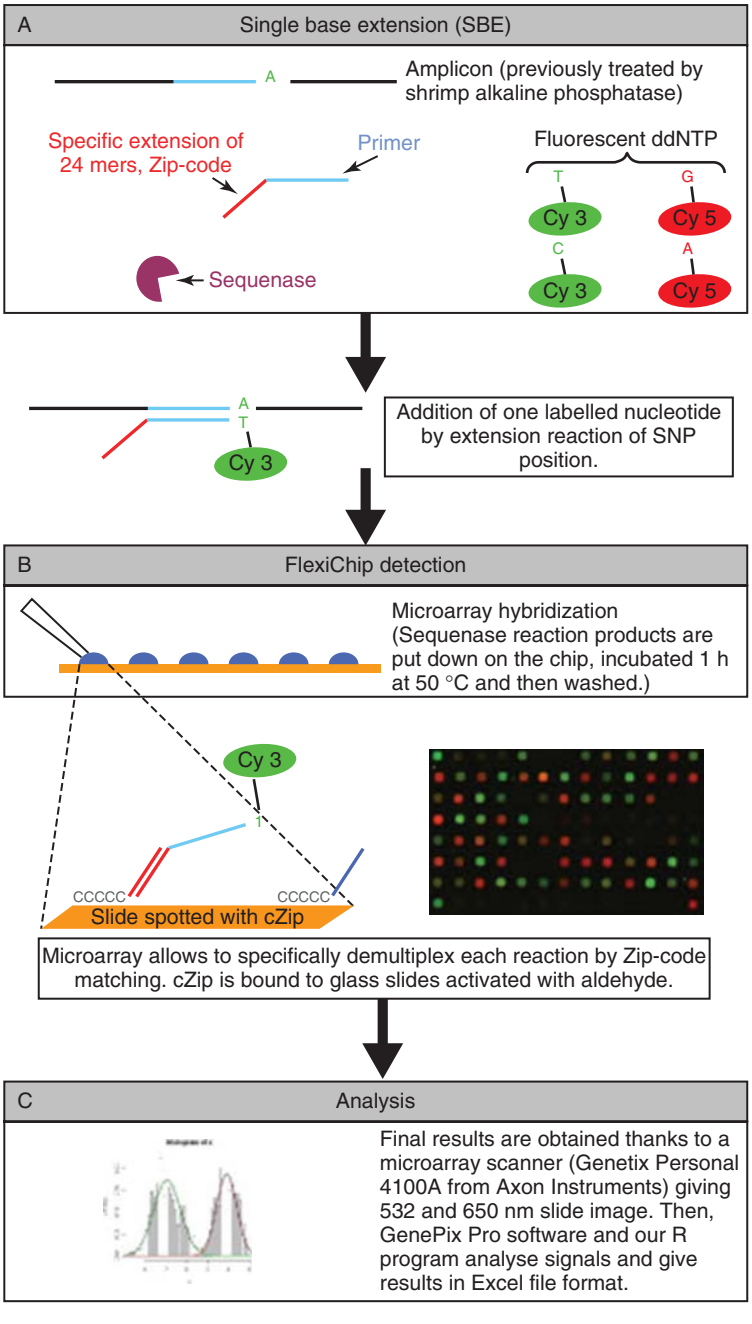
A
Single base extension (SBE)
A
Amplicon (previously treated by shrimp alkaline phosphatase)
Specific extension of 24 mers, Zip-code
Primer
Fluorescent ddNTP
T
Cy 3
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Cy 5
C
Cy 3
A
Cy 5
Sequenase
A
T
Cy 3
Addition of one labelled nucleotide by extension reaction of SNP position.
B
FlexiChip detection
Microarray hybridization (Sequenase reaction products are put down on the chip, incubated 1 h at 50 °C and then washed.)
Cy 3
T
CCCCC
CCCCC
Slide spotted with cZip
Microarray allows to specifically demultiplex each reaction by Zip-code matching. cZip is bound to glass slides activated with aldehyde.
C
Analysis
Final results are obtained thanks to a microarray scanner (Genetix Personal 4100A from Axon Instruments) giving 532 and 650 nm slide image. Then, GenePix Pro software and our R program analyse signals and give results in Excel file format.

Figure 4.4 Detection of antimalarial drug resistance in *P. falciparum* using an SNP typing DNA microarray (FlexiChip) [181]. Permissions: http://www.malariajournal.com/content/8/1/229/figure/F1.

are listed here (Table 4.7). Most recently, Brinkman *et al.* [190] described a DNA microarray-based on the CombiMatrix microarray platform for the detection of fecal indicator bacteria (*Enterococcus faecium*), waterborne pathogens (*C. parvum, Cryptosporidium hominis*), and biothreat agents (*Bacillus anthracis, Francisella tularensis*). The study aimed to develop a detection assay capable of detecting the pathogens without the need for target amplification. Having probes targeted against the ribosomal genes, the system managed to detect genomic DNA originating from cultures of 10^7 organisms. To model real sample detection, the authors spiked 100 ng genomic DNA in purified and concentrated tap water samples, and showed successful detection. They mention the detection of 20 copies in of *F. tularensis* genomic DNA in experiments investigating serial dilutions but give no further data to qualify the claim. Lee *et al.* [191] took a more focused approach in the detection of six species and four genera of the most important protozoan parasites. They designed a low-density DNA oligonucleotide microarray with probes against the small subunit rRNA genes; the targets were amplified using a conventional PCR and post-labeling. Interestingly, the authors tested the array performance in simulated environmental samples in which DNA was mixed with genomic DNA from *C. parvum*. Here, the authors could achieve a detection limit of 1000 copies of the SSU rRNA gene. The authors give a positive outlook on the use of the system in environmental monitoring, but follow-on studies are not known. In 2004, Wang and colleagues described a DNA oligonucleotide microarray for the detection of *Entamoeba histolytica*, *Entamoeba dispar*, *Giardia lamblia*, and *C. parvum* [192]. Here, the target DNA was amplified in a multiplex PCR reaction targeting a variety of non-rRNA genes (except for *C. parvum*) and genotyped using the several oligo probe sets on the microarray. The PCR products were directly labeled by Cy3-dCTP. Very low limits of detection could be achieved down to a level of five *G. lamblia* trophozoites. Also, the authors claimed the capability of the array to specifically detect co-infections, as demonstrated for 10 : 1 mixtures of two *G. lamblia* strains. As an early example, Straub *et al.* [193] developed and used a DNA microarray to discriminate various *Cryptosporidium* strains by genotyping *hsp70* in environmental isolates. The DNA microarray performed well in typing *C. parvum* strains, *C. wrairi, C. meleagridis, C. felis,* and *C. baileyi* and was highly regarded regarding its impact on regulatory compliance and epidemiology.

4.6.3
Other Parasites

Interestingly, the literature base for other human parasite testing using DNA microarrays is relatively limited. Two examples have been identified for human pathogenic microsporidia [195] and *Toxoplasma* [194]. Bahl *et al.* designed an oligonucleotide DNA microarray for genome-wide expression profiling for *Toxoplasma* and SNP genotyping of *Toxoplasma gondii* and *P. falciparum* to be used as a research tool by the *T. gondii* research community. Wang and colleagues used a conventional spotted DNA oligonucleotide microarray and PCR target

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Table 4.7 DNA microarrays for human parasite and protozoan pathogen detection.

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[194]	US	*T. gondii*, *P. falciparum*	Pure cultures	DNA isolation, fragmentation biotin-14-dCTP labeling	Affymetrix Toxoplasma gondii GeneChip	n/a
[179]	CH	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Blood samples	gDNA extraction, PCR, fragmentation, PCR primer extension fluorescent labeling	Nested PCR, four color primer extension, spotted multiwell oligo array, fluorescence detection	No clear data: sample prep., 1.5 h + hybridization, 42 min washing, readout, data analysis
[190]	US	*C. parvum,C. hominis*, *E. faecium*, *B. anthracis*, *F. tularensis*	Pure cultures, spiked tap water concentrates	gDNA extraction (MasterPure DNA purification kit (Epicenter Biotechnologies, Madison, WI, USA) from 10^7 cells. Labeling Cy3-dCTP using BioPrime DNA labeling system (Invitrogen)	CombiMatrix 12 and 4 × 2 K CustomArray	No clear data: sample prep., 13 h + hybridization, washing, readout, data analysis
[185]	UK	*P. falciparum*	Cultures	Whole genome amplification (multiple displacement amplification (MDA)), fragmentation, end labeling	Affymetrix scrMalaria arrays, microarray-based comparative genomic hybridization (CGH)	n/a
[175]	CH	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Cultures	gDNA extraction, PCR, fragmentation, PCR primer extension fluorescent labeling	Nested PCR, four color primer extension, spotted multiwell oligo array, fluorescence detection	No clear data: sample prep., 1.5 h + hybridization, 42 min washing, readout, data analysis

[177]	FR	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Blood samples	gDNA extraction, PCR, fragmentation, PCR primer extension fluorescent labeling	Nested PCR, four color primer extension, spotted multiwell oligo array, fluorescence detection	No clear data: sample prep., 1.5 h + hybridization, 42 min washing, readout, data analysis
[191]	CA	*Acanthamoeba* spp. *B. hominis, C. parvum, E. histolytica, G. intestinalis, N. fowleri, N. gruberi*	gDNA, pure cultures	gDNA extraction, PCR, post PCR fluorescent labeling	Spotted oligo array, fluorescence detection	n/a
[178]	CH	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Blood samples	gDNA extraction, PCR, fragmentation, PCR primer extension fluorescent labeling	Nested PCR, four color primer extension, spotted multiwell oligo array, fluorescence detection	No clear data: sample prep., 1.5 h + hybridization, 42 min washing, readout, data analysis
[176]	CH	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Blood samples	gDNA extraction, PCR, fragmentation, PCR primer extension fluorescent labeling	Nested PCR, four color primer extension, spotted multiwell oligo array, fluorescence detection	No clear data: sample prep., 1.5 h + hybridization, 42 min washing, readout, data analysis
[181]	FR	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Blood samples	QIAamp DNA Mini Kit	Spotted oligo array (zip code/FlexiChip), fluorescence detection	No clear data: sample prep., 1 h + hybridization, 45 min washing, readout, data analysis

(*continued overleaf*)

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Table 4.7 (*Continued*)

References	CC	Target species	Sample	Preanalytics	Detection	TTR
[193]	US	*Cryptosporidium* spp., *C. parvum*	Pure cultures (aiming for water)	gDNA extraction, fluorescent PCR labeling	Spotted oligo array, fluorescence detection	n/a
[195]	US	*E. bieneusi*, *E. cuniculi*, *E. hellem*, *E. intestinalis*	Pure cultures from culture-enriched isolates, urine, fecal specimens, and mucosal aspirates	gDNA extraction, fluorescent PCR labeling	Spotted oligo array, fluorescence detection	No clear data: sample prep., 1 h + hybridization, washing, readout, data analysis
[192]	US	*E. histolytica*, *G. lamblia*, *C. parvum*,	Pure cultures (aiming for water)	gDNA extraction, fluorescent PCR labeling	Spotted oligo array, fluorescence detection	No clear data: sample prep., 1 h + hybridization, washing, readout, data analysis
[189]	CN	*P. falciparum (drug resistance, pfdhfr, pfdhps, pfcrt, pfmdr1, pfATPase)*	Cultures, dried blood spot	gDNA extraction, PCR, fragmentation, post PCR fluorescent labeling	Spotted oligo array, fluorescence detection	n/a

amplification to detect various Enterozytozoon species. With a demonstrated detection limit of 10 spores, the authors tested the microarray in a clinical setting. Here, from 20 fecal samples from AIDS patients, 12 were microsporidia positive with 11 having mixed infections. While the study is already from 2005, it clearly demonstrates the applicability of such assay to clinical samples and provides the ground for further investigations with a clinical or epidemiological focus.

4.7 Conclusion an Outlook

For almost 25 years, DNA microarray technology has undergone remarkable developments spawning a plethora of scientific studies and commercial developments focusing infectious diseases, pathogen detection, and well beyond. As the design and implementation of content for DNA microarrays, that is, the design of probes for molecular targets, as well as the readout technology became routine, it increasingly turned out that the technical bottleneck for diagnostic DNA microarray development lies in the preanalytical steps. Sampling, nucleic acid extraction, and possibly amplification are crucial requirements for any molecular assay and, of course, also including DNA microarrays. While the technologies for the individual process steps exist, the integration in sample to answer formats that allow routine clinical use remains a major challenge, which so far has been solved only in a few cases. Finally, clinical relevance, market adoption, and cost models for reimbursement are among the key factors for the success of diagnostic DNA microarray assays for infectious diseases. In addition, as integration of microarray assays is well under way in a number of cases, DNA microarray technology is tackled from various ends. With regard to integration and molecular assay design, for example, advanced electrochemical DNA biosensors are much simpler and are gaining ground in the commercial [196] and academic sector [197]. However, DNA microarrays can be understood as complex DNA biosensors to which they are superior because of their multiplexing capability for ultra parallel analysis. Nevertheless, to the top end of complexity and deep molecular analysis of a sample, next-generation sequencing is increasingly taking over from DNA microarrays. While developments for clinical microbiology and outbreak detection are taking off recently [198, 199], examples of applications in routine clinical diagnostics and point-of-care detection are yet to be demonstrated. Here, DNA microarrays still have an advantage and will certainly be further used to drive establishing molecular diagnostics in the clinical routine for improved diagnostics and better treatment of infectious diseases.

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5
MALDI-ToF

Stefan Zimmermann

5.1
Introduction

Identification of bacteria by mass spectrometry has revolutionized microbiology in routine diagnostic laboratories within the last years. Many different techniques of mass spectrometry have been used in basic protein research approaches. Procedures such as ESI-ToF (electron spray injection time-of-flight) and SELDI-ToF (surface-enhanced laser desorption ionization time-of-flight) mass spectrometry, or QMSs (quadrupole mass spectrometry) have been developed over the last two decades. But only MALDI-ToF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry was pushed forward as a simple-to-use, reliable, and cheap technology and succeeded in the routine lab.

Mass spectrometry was used already in the 1970s as a new method to identify bacteria [1]. These early papers were mainly published in specialized journals for mass spectrometry and were not recognized by the broader scientific community.

To analyze a macromolecule by mass spectrometry, the protein has to be ionized and volatilized by a laser shot. Ionization of molecules by a laser beam includes the risk of the protein breaking down into fragments and losing its tertiary structure. This problem was solved in 1985 by two German scientists, Franz Hillenkamp and Michael Karas. They reported a method that uses small organic compounds as a matrix, protecting the molecules from this damage [2]. In the same year, Koichi Tanaka from Japan presented data where he used a mixture of glycerol and fine metal powder as a matrix. With these tools, he was able to ionize substances with a laser without structural losses. He published the method as soft laser desorption (SLD) after he had filed a patent application [3]. In 2002, Koichi Tanaka was awarded the Nobel Prize in Chemistry, together with John Bennett Fenn and Kurt Wüthrich (the latter for work in NMR spectroscopy), for his invention. Franz Hillenkamp was not considered in the distinction, although he was the first one to publish the method. He had already expanded the application of the new method to a large variety of molecules and higher masses in the year of Tanaka's first publication [4].

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It took some more years before the innovative method became routine in the microbiology labs. In 1994, Cain and colleagues used α-cyano-4-hydroxy-cinnamon acid as matrix molecule. They were able to record specific protein spectra which allowed typing of bacteria on a species level [5]. They already had the vision that the new method could shorten the time for pathogen identification dramatically. In addition, it might have the power to allow direct identification of bacteria from blood or urine without a culturing step. Holland and colleagues were the first to use a database approach to determine bacterial species [6]. They recorded different bacterial spectra of known species and then tested blinded strains with mass spectrometry. They still had to compare the generated spectra to the recorded ones by the bare eye. Each pathogen had specific peaks in the spectra, which allowed its identification. This was the beginning of chemo-taxonomy.

In the following years, a number of studies proved the applicability of the new technology in different bacterial genera. Haag and colleagues tested it in the fastidious genus *Haemophilus*, where typing had a number of problems because of biochemical reactions [7]. In the beginning, the method spread slowly, and most papers were published not before the turn of the millennium [8–10]. The first study analyzing a representative number of isolates from clinical samples was published in 2009 by Seng [11]. The findings of Raoult's lab in Marseille and the Limbach lab in Heidelberg (later published in [12]) presented at the 18th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) in 2008 in Barcelona opened the field to a broader public. All the scientists used MALDI-ToF technology, whereas the other methods, such as SELDI-ToF or SLD, did not succeed in the routine lab.

5.2 MALDI-ToF Technology

For a century, typing of bacteria was mainly based on biochemical reactions measured in liquid or semiliquid cultures. Different enzymatic substrates were provided to the bacteria of interest in a number of test tubes, which had to be incubated for several hours or even days. The biochemical characteristics of a single strain had to be read and documented manually and compared to databases or tables, for example, for enterobacteriaceae, a table spanning six pages in Bergey's Manual [13]. Later commercial systems became available in plastic strips or preformed panels, which were easier to use but still time consuming. If the viability of the organism was weak, for example, after isolation from a patient with antibiotic therapy, or it was of metabolic arrest, the identification was difficult and often impossible.

The success of mass spectrometry in bacterial identification is based on the simplicity of the method and the rapid availability of results. Grown bacteria are identified in the mass spectrometer by typical protein mass spectra. The mass spectrum of immobilized, intact bacteria is determined under defined conditions

and compared to a database of thousands of spectra of known bacteria. Identification of bacteria on the basis of their chemical – mainly protein – compounds was named *chemotaxonomy*. As neither a further incubation of the microorganisms nor a time window for the metabolization of biochemical substrates is necessary, the results of MS identification of bacteria is available within minutes. The bacterial mass has to be immobilized on reusable steel targets or disposable silicon-based targets, which nowadays are commercially available in different sizes (48, 96, or 384 spots per target). The mass is air-dried on the target and then mixed with a matrix of a low molecular weight compound. If no matrix is supplied, mass spectrometry in the beginning was only accurate for molecules smaller than 1500 Da [14]. Karas and Hillenkamp worked with 2,5-dihydrobenzoic acid to expand the mass range to 100 000 Da. Beavis and Chait [15] already in 1989 suggested hydroxy cinnamonnic acid derivatives as matrix compounds, which is still the most common matrix in commercial systems. The use of this compound resulted in naming the whole method as MALDI-ToF analysis. Though the possibility opened by Hillenkamp expanded the range of molecular masses up to 100 kDa, the use of the new technique in the early studies in the field of microbiology focused on lower masses between 500 and 2000 Da [16]. The range, which was later on successful in many labs of the world, between 2000 and 20000 Da was first described by Krishnamurthy in 1996 [17]. All the commercial systems and databases available today use this range.

The workflow to identify bacteria by MALDI-ToF technology is simple and reliable. Intact, whole bacteria are transferred to the target, mixed with the matrix, air-dried, and then transferred to a mass spectrometry instrument. The mass spectrum of the unknown bacterium is generated under defined conditions and compared with the mass spectra of known bacteria in a database. The database entry with the best match in the database to the generated spectrum is displayed as the result of the analysis. Commercial systems optimize the search and compare the strategy by several algorithms, which are part of the trade secret and not revealed to the customer. These procedures mostly create a score, which offers the user a confidence interval for the database hit. The score value helps in classifying the result either on a species or on a genus level. The workflow is displayed in Figure 5.1.

Remarkable is the amount of bacteria that is necessary for the identification. Commercially available identification kits for pathogenic bacteria (e.g., API® strips, Biomerieux A/S) require 5×10^8 to 1×10^{10} bacteria, depending on the kit and the investigated genus. For MALDI-ToF identification, a bacterial mass of 5×10^5 to 1×10^6 is sufficient. This means a 3 log difference in the amount of pathogens needed. Even if the generation time for fast-growing bacteria is less than an hour, this means a reduction of the time-to-result in identification of several hours. For slow-growing bacteria, the resulting difference can even be days. In a clinical setting with patients suffering from severe infections or even septicemia, hours can decide between life and death [18, 19].

Three different protocols are common to transfer bacterial masses to the MALDI target. The most common one is the simple smear preparation.

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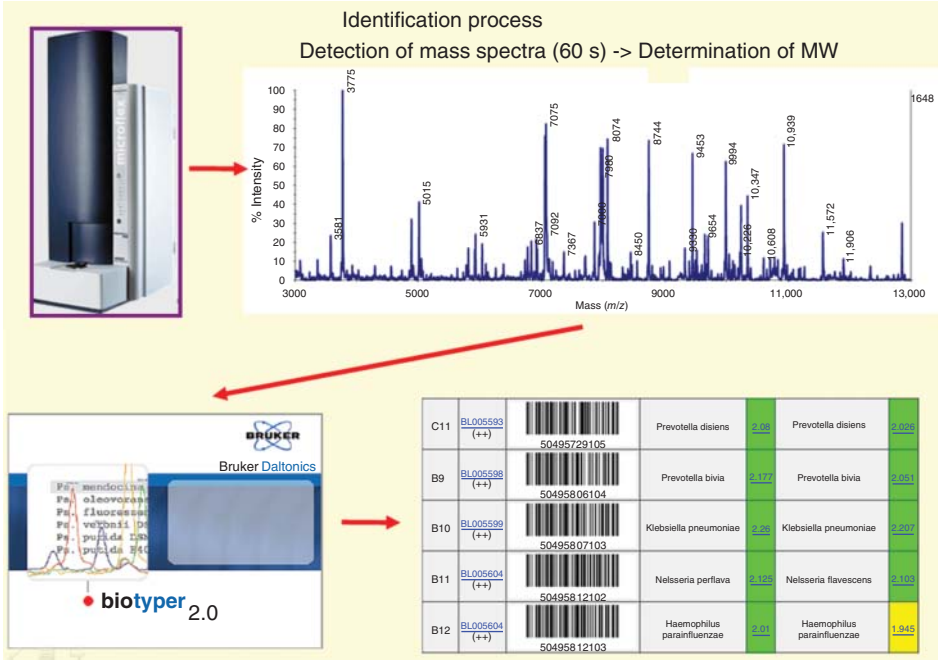
Identification process
Detection of mass spectra (60 s) -> Determination of MW
% Intensity
Mass (m/z)
Bruker Daltonics
biotyper 2.0
C11 BL005593 (++) 50495729105 Prevotella disiens 2.08 Prevotella disiens 2.026
B9 BL005598 (++) 50495806104 Prevotella bivia 2.177 Prevotella bivia 2.051
B10 BL005599 (++) 50495807103 Klebsiella pneumoniae 2.26 Klebsiella pneumoniae 2.207
B11 BL005604 (++) 50495812102 Neisseria perflava 2.125 Neisseria flavescens 2.103
B12 BL005604 (++) 50495812103 Haemophilus parainfluenzae 2.01 Haemophilus parainfluenzae 1.945

Figure 5.1 Principle of MALDI identification workflow.

A bacterial colony is tipped with a 1-µl loop, a pipette tip, or a tooth pick, and a minimal amount of the colony is transferred to the target plate. It is applied to the target position by smearing it directly on to it. Then the bacteria are air-dried and overlaid with a specific matrix. In commercial systems nowadays α-cyano-4-hydroxy-cinnamic acid (HCCA) is recommended for standard procedures. The matrix again has to be air-dried for a few minutes. Then the target spot is ready for the analysis of its mass spectrum. Normally, targets with 48 or 96 detection spots are used in the microbiology routine lab, therefore analyzing a single spot is an exception.

In cases where the smear spectrum is not meaningful to identify the pathogen – as the number of mass peaks is insufficient to generate a hit in the database or no mass peaks are detected at all due to insufficient protein extraction thru the intact bacterial cell wall – an extraction protocol is used. The bacteria are dissolved in water, mixed with ethanol, and pelletized. The dried pellet is mixed with formic acid, and then acetonitrile is added to extract proteins and peptides. The mixture is centrifuged again, and the proteinaceous supernatant is transferred to the MALDI target. The measurement is the same as for the smear preparation.

Bacteria-containing spores often generate two different mass spectral patterns: one of the viable cells in the mixture, and a different one for spore cells. This mixed pattern does not produce sufficient concordance with the database entries and often results in inconsistent results.

If highly infectious biological material is to be analyzed (e.g., *Brucella* or *Francisella*), a third protocol containing an inactivation step is available. The bacteria are resuspended with 80% trifluoroacetic acid (TCA) and, after an exposure time, mixed with water and acetonitrile. The suspension is pelletized, and the proteinaceous and noninfectious supernatant is analyzed as described.

The prepared target is inserted into the measurement chamber. As a high-voltage laser is used, the instrument operates at a high vacuum of about 2×10^{-6} mbar. A 20-Hz laser shoots on the target spot, and bacterial molecules embedded in the matrix are desorbed and ionized. An electric tension of 10 kV is applied, and the protonized molecules are accelerated in the vacuum tube. At the end of the tube, a linear detector analyzes the impact of the molecules. Smaller molecules, or smaller masses, fly faster and are detected earlier at the detection shield, while larger molecules fly more slowly and therefore have a longer time of flight (Figure 5.2). As the acceleration in the electric field depends on the charge of the molecule, the resulting unit is not absolute mass but mass per charge (m/z).

Up to 40% of the protein mass of bacteria are highly conserved ribosomal proteins. So the proteinaceous spectrum of a specific bacterium is relatively stable independently of the agar on which it was grown. Therefore, the spectrum of a bacterium generated, for example, from a sheep blood agar plate differs not significantly if grown on chocolate agar or MacConkey agar. This robustness of the system was to key to the success of the method in the daily lab routine, as the database must not contain many different entries of every bacterium grown on many different sources. For other new promising technologies of microbe identification, such as Raman spectroscopy, for example, this is still a problem limiting their daily use in routine procedures.

So, different bacterial species give different mass spectra, these are compared with the database, and a result list is displayed by the instrument (see example in Figure 5.3).

5.3 Bacterial Identification Using Mass Spectrometry

Only a few years after the matrix-based mass spectrometry technology was described, the first papers appeared in the field of microbiology. In the beginning, it was used to characterize new metabolites, as shown by Erhard and colleagues for cyanobacteria [20] or proteomes of specific compartments, for example, the membrane proteome of *Escherichia coli* [21]. Another focus in the early years was deeper analyses of posttranslational modifications in bacteria, either investigating changes in ribosomal proteins of *E. coli* [22] or proteome differences in fastidious organisms such as *Helicobacter pylori* to better identify this human pathogen [23], which was just discovered a few years earlier in the mid-1980s [24].

After the first researchers had recognized the potential of the new technology to generate databases for the routine identification of bacteria, different labs started

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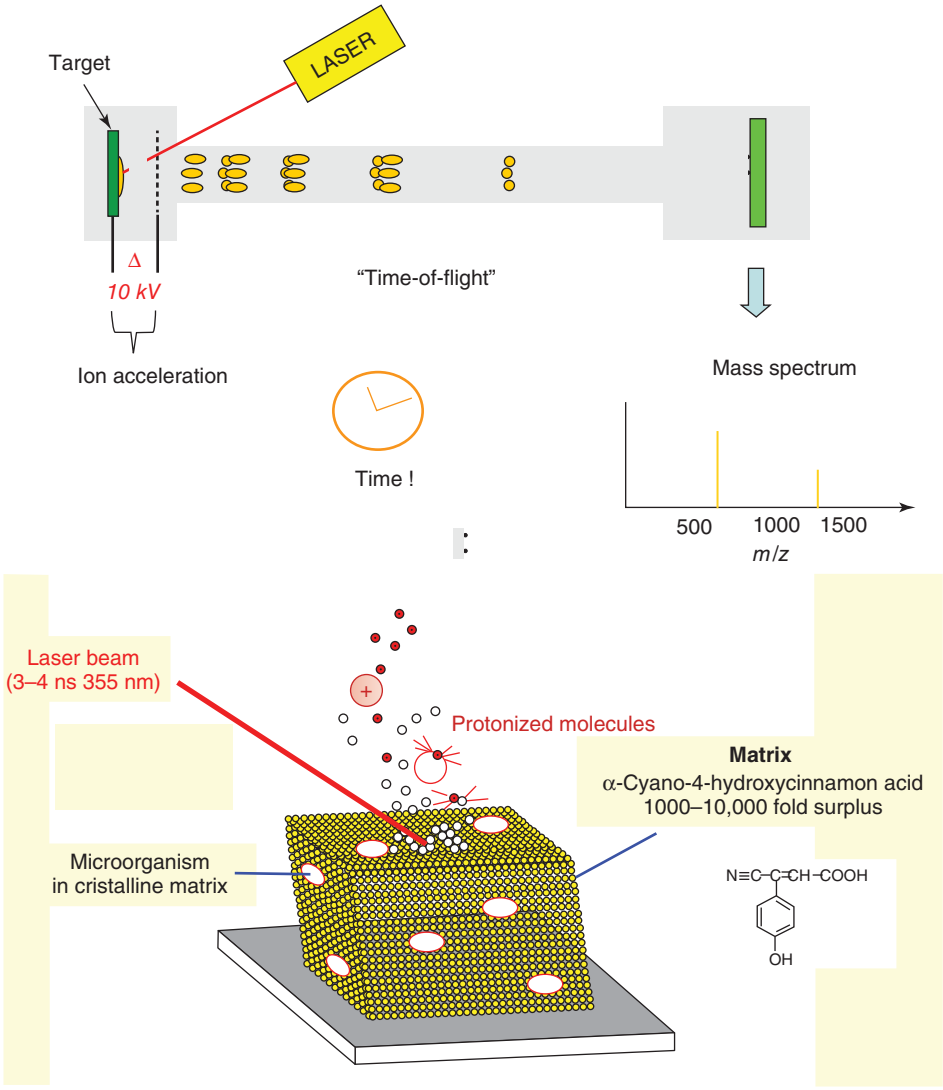

Figure 5.2 Matrix-assisted mass spectrometry.

to investigate the influence of growth conditions and different agar plates on the resulting spectra [25, 26]. Also, factors such as the pH, temperature, and growth rate during fermentation were analyzed [27]. To finally prove the reliability of the method for bacterial identifications, also interlaboratory comparisons were performed, to make sure that bacterial strains give the same results in different labs when using mass spectrometry as the method of choice for typing [28].

After it was clear that the conditions of the medium had limited or no influence on MALDI spectra, the door was far open to use the new method in routine

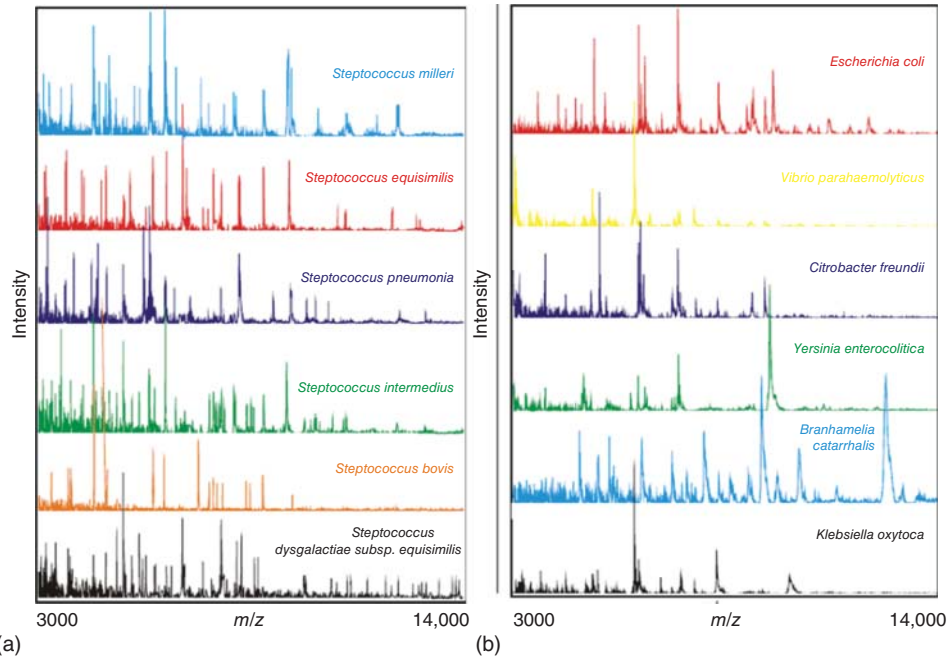

Figure 5.3 (a,b) Mass spectra of different bacteria.

approaches. Therefore, a huge number of genus and bacterial families were investigated, and databases were generated. Carbonelle and colleagues were the first to identify a broad range of micrococcaceae, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, or *Micrococcus luteus.* They generated 45 spectra of known micrococcaceae, but did not use the whole obtained spectrum of each strain. Instead, they identified up to 14 species specific peaks. The generated database was challenged by testing 196 micrococcaceae isolates. All of them were correctly identified by the system, but these database entries were not stable against changes in the culture conditions. If the growth medium was changed or the duration of culture was modified, the hit rate in the database decreased [29]. Later, Dubois and colleagues tested 152 *Staphylococcus* isolates in the commercial Biotyper database (Bruker Daltonics, Billerica, US). They identified the strains by two different molecular methods: on the basis of the sodA gene (superoxide dismutase A), and in parallel using the 16S rRNA gene. The mass spectra results showed a higher identity to the 16S-based phylogenetic tree than to the sodA based one, which is not surprising as ribosomal proteins dominate the proteome spectrum in the used mass range between 2000 and 20 000 Da [30]. A similar approach was tested a year later by Spanu *et al*. [30a]. They tested 450 staphylococcus strains isolated from blood cultures against the Biotyper database, but used the rpoB gene for molecular typing. They found that 99.1% of coagulase negative strains showed identical results and all *S. aureus.* These results were confirmed when Rajakaruna *et al.* tested more than 100 *S. aureus* isolates

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against another commercial database of the MicrobeLynx® system. They found 97% of the strains were correctly identified on a species level [31]. In Dubois' study in 2010, only 93% of non-aureus strains were correctly identified on a species level. After three missing species were added to the Biotyper database, the ratio climbed to 97.4% [32]. This certifies one of the big advantages of the new technology. If new species in a known genus were discovered or known bacteria were identified as human pathogens, for example, in immunocompromised patients, their spectra can be easily added to the databases and the range of detectable microorganisms expanded.

The bacterial family of the streptococcaceae is also very important as human pathogens. Even after the enterococci were separated in 1994, still more than 50 species remained in the genus *Streptococcus.* As the biochemical identification of streptococci was often unsatisfactory due to their slower growth and their fastidious attitudes on different culture media, the new method raised the interest of microbiology specialists in the field very early. Already in 2005, Rupf *et al*. published their first study on the identification of *Streptococcus mutans*, the most important contributor to tooth decay, which may be the most common infectious disease in humans [33]. Especially, the two closely related species *S. mutans* and *Streptococcus sobrinus* both cohabitate the mouth and can contribute to the disease, they cannot often be distinguished. First a MALDI database for four non-mutans streptococcus groups (*Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus salivarius*, and *Streptococcus sanguis*) and the mutans group was generated, and then challenged with 175 *S. mutans* and *S. sobrinus* isolates. To make sure that the species identification was correct, the strains were not only typed by 16S rRNA PCR sequencing, a method known to have a low discriminatory power in the genus *Streptococcus*, but also identified by species-specific PCRs. Comparison to the new database showed 100% correct identification with MALDI-ToF MS. A broader approach by Friedrichs *et al*. tried to identify viridans streptococci, mainly *S. mitis*, *S. anginosus*, or *S. salivarius*, with mass spectrometry, showing convincing results again compared to biochemical methods and different PCR approaches [34]. Blondiaux's study to determine streptococci from clinical specimen by MALDI-ToF technology resulted in a hit rate of only 65% [35], while a similar approach by Eigner's group showed a 94% success rate [12]. It turned out that the accuracy of the method in streptococci depends, among others, on the portion of *Streptococcus pneumoniae*, *S. mitis*, and *S. oralis* in the tested isolates. These three species seem to be difficult to differentiate by mass spectrometry; at least for the first two this holds also true for 16S rRNA PCR sequencing [36].

Just recently, a paper was published showing that the discrimination between these closely related species is possible when the mass spectra are sorted correctly and additional algorithms are used for database comparison [37]. The genus *Enterococcus* was first analyzed by Seng *et al*. in 2009. Of 84 enterococcus strains, 71 were isolated on a species level (84.5%) and the other 13 on the genus level [11]; none of the isolates was misidentified. In recent years, more detailed analyses have been published. The first was when Werner investigated 199 group D streptococci, of which 101 turned out to be of the two most important human

pathogenic species *Enterococcus faecalis* and *Enterococcus faecium.* The Biotyper results were confirmed by species-specific PCR and by an additional multilocus sequence typing (MLST) [38]. At the same time, Fang published his analysis on the same enterococcus species but added isolates of *Enterococcus casseliflavus* and *Enterococcus gallinarum*, both known for their natural resistance against vancomycin (induced by the *vanC* gene). They compared both common commercial systems – the Bruker Biotyper® and the Vitek MS® (Biomerieux, Marcy, France) – and confirmed that both instruments detected all 132 samples correctly at the species level [39].

Although Gram-positive cocci play a more important role in medical microbiology, Gram-positive rods can also be identified by mass spectrometry.

Barbuddhe and colleagues generated a database using the six most common *Listeria* species. The database was tested against 138 different *Listeria* strains, which proved excellent results on a species level. They defined species-specific peaks in a mass range below 8000 Da for the reliable identification of closely related strains. A combination with pulse-field gel electrophoresis (PFGE) allowed even the discrimination of serotypes [40].

The genus *Bacillus* is one of the most complex and difficult ones in the taxonomy of pathogenic bacteria. *Bacillus anthracis*, the cause of anthrax, is most frightening as a human pathogen and as a biological weapon. Over 200 species have been part of this genus before it was regrouped, and nine additional genus, like *Brevibacillus* or *Paenibacillus*, has been generated. Lasch and colleagues took on the burden to set up a database for the Bacillus complex [40a]. For work with *Bacillus spp.* a special biosafety level is necessary, therefore the authors used the TCA extraction methods. Database entries of 25 different species were generated and tested for their accuracy. As many of the species cluster in closely related subgroups, for example, the *Bacillus cereus complex* or the *Bacillus subtilis complex*, specific peaks were analyzed in the gel view mode of the spectra to differentiate also species closely related in taxonomy. In addition, the authors created specific algorithms such as unsupervised hierarchical cluster analysis (UHCA) and supervised artificial neural network (ANN) to optimize the discriminatory power of the analysis. These addendums are not yet available for routine diagnostics.

Coryneform bacteria comprise another huge family of Gram-positive rods in medical microbiology. A number of new genera have been defined over the last years, and several species were newly described in the genus *Corynebacterium* [41]. A problem with thick-walled Gram-positives in mass spectrometry sometimes appears: the reliability of specific spectra is difficult to reproduce because of the problem that the amount of proteins released for the time-of flight analysis is limited. Therefore, the simple smear preparation technique frequently results in low accuracy of spectral identification. Theel and colleagues overcame the problem by using a simple "on target" preparation of the samples. They used the smear preparation and added 1 µl of 70% formic acid to extract more proteins. After the acid was air-dried, cinnamic acid as matrix was added, and the spot tested in a Bruker microflex® was compared to the Biotyper® database. More than 92% of

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the tested corynebacteria were identified on a species level, and less than 4% were missed on the genus level [42]. Farfour *et al*., to date, tested the largest collection of coryneforms. They analyzed 40 *Corynebacterium diphtherie*, 13 *Corynebacterium pseudodiphtheriae*, 19 *Corynebacterium ulcerans*, and 270 other strains on a Bruker microflex® using their own Andromas® database system. When compared with this database, 98.5% of the isolates were identified on a species level and only 1.2% were missed on the genus level [43]. In Spain, an analysis of 52 *Corynebacterium striatum* isolates proved that even this difficult species can be discriminated by MALDI-ToF compared to a multisequencing approach using *its1*, *rpoB*, and *gyrA* genes [44]. Mass spectrometry was recently also described in the identification of coryneform bacteria. De Vreese *et al*. identified a small number of *Actinomyces neuii* strains using MALDI-ToF MS and compared with conventional methods and sequencing. They concluded that MALDI-ToF MS allows rapid and reliable identification to species and even subspecies level [45].

The most important family of Gram-negative bacteria is the enterobacteriaceae. These species are the most frequent cause of urinary tract infections and nosocomial pneumonia. It is one of the largest bacterial family with more than 50 different genera and hundreds of species, including frightening species such as *Yersinia pestis*, the cause of Black Death, and *Salmonella typhi*, inducing typhoid fever. Biochemical differentiation of this huge taxon has often been a problem. Therefore, Lynn *et al*. already in 1999 published a study in which they identified different enterobacteriaceae, including *E. coli* and *Salmonella* spp., by specific peaks within the mass spectra [46]. The first elaborate study of identification of enterobacteriaceae from routine samples using mass spectrometry was published by Seng in 2009. From 396 isolates, most of them members of the genus *Citrobacter*, *Enterobacter*, *Klebsiella*, and *Escherichia*, only 6 (1.5%) was not identified correctly on the species level when compared with conventional diagnostic methods [11]. Eigner at the same time performed a study with as many as 426 different enterobacteriaceae, all isolated in normal lab routine. MALDI-ToF identified all strains correctly on the genus level and 96% down to the species level [12]. These encouraging results inspired others to elaborate the new method on a single genus and test its discriminatory power. Usefulness of mass spectrometry in the genus *Yersinia* was already shown in 2006 by Mazzeo *et al*. [47]. In this early study, five environmental isolates were differentiated by MS. Later, Ayyadurai investigated a large set of *Yersinia* strains with 12 different species, including the dangerous *Y. pestis.* All 40 isolates were identified on the species level, and MALDI-ToF was found to be advantageous over other methods [48]. Mazzeo in his approach on foodborne pathogens not only investigated *Yersinia* but also the genus *Escherichia.* He was able to differentiate five different *Escherichia* species [47]. In addition, he found serotype-specific biomarkers to discriminate the enterohemorragic *E. coli* O157:H7 strain (EHEC). EHEC can cause non-bloody and bloody diarrhea and the hemolytic uremic syndrome (HUS). HUS is a major cause of acute renal failure in children, and *E. coli* O157:H7 is the predominant serotype that can cause HUS [49]. Only a few years ago, *Salmonella* was the most frequent bacterial cause of diarrhea in humans.

There was a long debate on the taxonomy of this genus, which historically has had more than 200 species. It ended in the commitment of only two species groups: *S. typhi* and *S. enterica.* All the other species were degraded to the subspecies level, because their biological relationship was so close. Leuschner was the first researcher to investigate the complex genus with MALDI-ToF technology. He successfully analyzed 22 different subspecies and defined specific peaks for the different subspecies. To simplify the analysis for unknown subspecies, he had used computational spectrum analysis already 10 years ago [50]. An even more detailed analysis was done by Dieckmann, who classified 126 Salmonella isolates, on a species and subspecies level using mass spectrometry. To optimize the spectra, he used sinapinic acid as an additional matrix. In addition to the excellent subspecies discrimination, the study defined nearly 60 species-identifying biomarkers for the Salmonella group [51]. In the large enterobacteriaceae family are many genera and species that are nonpathogenic for humans. But some species have been identified as rare human pathogens within the last years. The genus *Erwinia* is an interesting example. The genus is known for many years as a plant pathogen. The most investigated species *Erwinia amylovora* is the cause of "fire blight," a dangerous infection of apples and pears causing huge losses in pomiculture every year. Recently, there have been a few reports demonstrating that *Erwinia* can affect also humans. *Erwinia persicinus* was isolated as the cause of a urinary tract infection [52], while another *Erwinia* species, closely related to *Erwinia tasmaniensis*, was identified as the source of a cervical lymphadenitis in a patient in Korea [53]. Commercially available biochemical identification methods, such as API® or Vitek®, often misidentify *Erwinia* as *Pantoea species.* Mass spectrometry was shown to dissect not only the two related genera but also the discriminatory power to distinguish even the two closely related species *E. amylovora* and *E. tasmaniensis* [54].

Among the enterobacteriaceae, non-fermenting Gram-negative rods are important human pathogens causing an increasing number of infections. Especially multi- or even pan-resistant strains of *Pseudomonas aeruginosa* or *Acinetobacter baumannii* are an increasing cause of nosocomial infections and a real threat to hospital patients [55, 56]. Commercially available instruments for bacterial identification, such as Vitek® or Walkaway®, showed a higher misidentification rate for these bacteria compared to enterobacteriaceae [57]. When Eigner first investigated a larger sample set of non-fermenting rods using MALDI-ToF, he could demonstrate promising results. All tested 128 strains could be identified on a genus level, while only 102 (80%) were identified on a species level [12]. Over the last 5 years, some efforts has been made to enlarge the databases of commercially available mass spectrometers to optimize these results. Mellmann and colleagues also analyzed non-fermenting rods on a Bruker mass spectrometer using non-isolates from the daily lab routine. They tested even 248 different species out of 37 genera, and found a very good correlation on the genus level and already 86% on the species level [10]. They concluded that mass spectrometry was also less laborious and much faster than conventional methods. In a follow-up study, they could also show that the method has a high interlaboratory reproducibility

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of 99%. Sixty blind-coded strains were sent out to eight different labs in an international comparison, and only 6 of 480 samples were misidentified [58]. A detail pointed out in the first study was that mass spectrometry has an even higher discriminatory power than 16S-rRNA PCR in the *Burkholderia cepacia* complex. The latter is an important pathogen in children and adults suffering from cystic fibrosis. Therefore, it was consequential that Degand and colleagues used the MALDI-ToF technology in an approach to identify 559 isolates from cystic fibrosis patients [59]. In the first experimental run, the accuracy of the typing was 83–98% for the different genera. After re-engineering the database especially for the genera *Burkholderia* and *Ralstonia*, the accuracy increased to 94–100%. The conclusion of this elaborate study was that MALDI-TOF-MS is a powerful tool for rapid identification of non-fermenting Gram-negative bacilli.

Other non-fermenting rods, such as the genus *Vibrio*, were also targeted in mass spectrometry experiments. *Vibrio cholerae* is the most well-known species of the group, as it led to huge outbreaks in the second half of the nineteenth century. Dieckmann first described the use of mass spectrometry to identify *Vibrio* and *Aeromonas* strains making use of defined specific mass peaks. The taxonomic relationship seen in MALDI dendrograms correlated with the taxonomy tree based on *rpoB* gene sequencing [60]. Another interesting study by Gerdts' group investigated the *Vibrio* populations in the rapidly changing ecosystems of the German Bight on the background of increasing seawater temperature due to global warming. They isolated and typed a number of *Vibrio parahaemolyticus* and *Vibrio alginolyticus* species with close proximity to pathogenic pandemic strains combining mass spectrometry, PCR, and sequencing [61]. In Europe, *Vibrio* is a rare source of human diarrhea compared to another oxidase-positive genus *Campylobacter*, which has displaced *Salmonella* in the ranking of the most common bacterial enteric pathogen in recent years. Winkler *et al*. first used an attempt with specific mass peaks to differentiate the most frequent human pathogen [62]. A few years later, a Swedish group investigated more than 120 different Campylobacter isolates, human pathogens, and environmental strains, defined species-specific peaks, and set up a spectra database for routine diagnostics [63]. Rapid and accurate identification of Campylobacter species is highly relevant to public health and food safety. The method was successfully used on isolates of pediatric patients with severe diarrhea from a hospital in Burkina Faso to confirm the conventionally typed strains with mass spectrometry [64]. In addition, they were able to identify the species even though samples contained two mixed strains. For the genus *Haemophilus*, with its most pathogenic member *Haemophilus influenzae*, early approaches with MS were done by Haag and colleagues already in 1998 [7]. The smaller amount of bacterial mass for identification with MALDI-ToF shortened the time-to-identification of this important cause of meningitis. Later on, mass spectrometry was used in routine approaches to diagnose *Haemophilus* spp., but in addition all members of the HACEK group (*Haemophilus* spp., *Aggregatibacter* spp., *Cardiobacterium hominis*, *Eikenella*

corrodens, and Kingella kingae), which are known for their potential to induce endocarditis [65]. Recently, Bruin *et al*. demonstrated the usefulness of MS to differentiate between *H. influenzae* and the less pathogenic but closely related *H. haemolyticus.* In a study with as much as 277 isolates, again engineering of the database was the key to an excellent taxonomic discrimination of 99.6% [66]. A broad range of other fastidious bacteria were also investigated with mass spectrometry. A few of them are remarkable and should be mentioned in detail. Fournier *et al*. [67] reported the identification of *Bartonella species*, the agent of trench fever (*Bartonella quintana*) or cat-scratch disease (*Bartonella henselae*), using automated mass spectrometry. They investigated 17 different *Bartonella* species and engineered the database. In the next step, 39 blind-tested *Bartonella* strains could be identified correctly. Brucella is one of the major zoonotic diseases. Its laboratory diagnosis is difficult, time consuming, and hazardous, as it is the most frequent laboratory-acquired infection worldwide [68]. Two groups from the Friedrich Löffler Institute in Jena and Riems set up a mass spectral database for eight *Brucella* species, including *Brucella melitensis*, and several serovars. The quality and accuracy of the new database was confirmed by an interlaboratory comparison [69]. In some countries, a re-emergence of tularemia has been observed in recent years. Splettstoesser and Neubauer reported an outbreak of *Francisella tularensis* in Germany in 2007 [70]. This agent is also very hazardous and is classified as a biological weapon in the War Weapon Control Act. Therefore, handling of this pathogen in the lab should be reduced to a minimum. Seibold tested 40 *Francisella* strains and demonstrated that all were correctly identified in the system [71]. In addition, MALDI-ToF had the discriminatory power to subdivide the three *F. tularensis* subspecies, which is important because they have different hazardous potentials. *Leptospira* was the only genus of the spirochetes where mass spectrometry was used for identification; no attempts have been published till today for the genus Borrelia and Treponema. In his remarkable study, Djelouadji used 40 *Leptospira* strains of 19 different species to generate a database which now is able to identify cultured *Leptospira* isolates [72].

A number of approaches were started to identify members of the huge genus *Mycobacteria* by mass spectrometry. Researchers focused on two major problems. First is the mycobacterial cell wall, which is very thick and rich in mycolic acids, which makes it difficult to extract proteins for the analysis. The second problem is the hazardous potential of the *Mycobacterium–tuberculosis* complex group, which requires a special inactivation protocol to inactivate the microbes, as only a few labs in the world have mass spectrometers in the safety laboratory. Ten years ago, Hettick started with only six different mycobacteria and developed an extraction protocol based on TCA and acetonitrile to release a sufficient amount of proteins and small biomolecules (<2000 Da) from the mycolic acid envelope [9]. Lotz analyzed 311 strains belonging to 31 distinct species. In addition, he compared two standard mycobacterial media, the solid Löwenstein–Jensen agar (LJ) and the liquid MGIT medium (Mycobacteria Growth Indicator Tube). He found out that 97% of the isolates were correctly identified on a species level from LJ, while the

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success rate from MGIT was only 77%; but in both cohorts no misidentification occurred [73]. Saleeb generated a different database at the NIH and optimized the method for rapid and accurate identification of mycobacterial strains by mass spectrometry [74].

Anaerobic bacteria have always had an exceptional position in the microbiology laboratory. They only grow under specific anaerobic conditions, have specific differentiation schemes, and require specific handling. Therefore, it was a milestone when Shah reported in 2002 that proteomics of anaerobes could be performed on MALDI-ToF. As stated in the manuscript title, this opened "a new era in anaerobic microbiology" [75]. Shah's work focused on the genus *Prevotella*, and the use of MALDI-ToF in anaerobe microbiology was later expanded by Nagy for the genus *Bacteroides*. In her approach, 270 of 277 *Bacteroides* isolates were correctly identified on the species level [76]. Recently, Nagy's group added important information to the proteomic databases not only by characterizing a large number of *Propionibacterium acnes* strains but also providing the knowledge even to discriminate them on a phylogroup level [77].

After <10 years of MALDI-ToF research in clinical microbiology laboratories, nearly every genus containing human pathogenic bacteria can be identified by mass spectrometry. The knowledge of typing bacteria by their morphology and their biochemical profile has spanned a century. Proteomics and mass spectrometry have generated data in a routine and capable way in less than a decade.

5.4 Culture-Independent Rapid Identification of Clinical Pathogens

Parallel to the development to differentiate all the pathogenic microbes from cultured media, approaches were performed to save time-to-result with this technical milestone and develop culture-independent identification protocols.

Septicemia is a major cause of mortality in hospitalized patients worldwide, with lethality rates ranging from 30 to 70% [78]. Septicemia can be caused by a variety of different infectious agents. Early and adequate antimicrobial therapy correlates with positive clinical outcomes [18, 19, 79, 80]. Thus, rapid microbiological diagnostics are of high importance to improve the survival of patients. To date, blood culture has been the gold standard for diagnosis of bloodstream infections. Microorganisms present in a blood sample are enriched in a cultivation medium in blood culture bottles under continuous monitoring. When growth is detected, Gram staining is performed, which allows the first adjustment of antibiotic therapy. Subcultivation is needed for the identification and antimicrobial susceptibility testing (AST), which takes on average 12–48 h. Therefore, the results of the final differentiation and AST are often obtained too late to influence vital treatment decisions.

Conventional identification of bacteria by subculture and subsequent biochemical reactions take up to 48 h depending on the species and system used. In contrast, MALDI-TOF MS can be performed with minimal amounts of

bacteria and takes only a few minutes. The direct identification of microorganisms from a positive blood culture can significantly shorten the time for exact species determination, enabling earlier adjustment of the antimicrobial therapy.

Very early, different researchers attempted to save the time needed for plate culturing positive blood cultures by a direct identification approach using mass spectrometry. The first promising results were disclosed to the public by Kostrzewa at the ICAAC 2008 in Washington (Poster ICAAC/IDSA Washington 2008). The first peer-reviewed data were published by La Scola in 2009. They recommended a two-step centrifugation protocol for BACTEC® blood culture bottles (Becton Dickinson, Sparks, MD, USA). Using a low-speed centrifugation step first to remove erythrocytes and other cellular components, they pelleted the cultured bacteria in a second high-speed step. The pellet was then extracted with the formic acid/acetonitrile extraction protocol, and the proteinaceous supernatant was analyzed in a Bruker microflex mass spectrometer. With this protocol, 94% of Gram-negative bacteria in the blood cultures could be identified correctly in <1 h after the positive signal from the instrument [81]. The problem was that only a maximum of 67% of Gram-positive bacteria were identified correctly on the species level. Two pitfalls remained after the study: the weakness in identifying Gram-positives, especially viridans-streptococci, and the lack of detection of mixed cultures. But not only bacteria cause septicemia; also invasive fungal infections are an increasingly frequent etiology of sepsis in critically ill patients, causing substantial morbidity and mortality. *Candida* species are by far the predominant agent of fungal sepsis, accounting for 10–15% of healthcare-associated infections and about 5% of all cases of severe sepsis and septic shock, and are the fourth most common bloodstream isolates in the United States [82]. Therefore researchers also tried to directly identify Candida species from positive blood cultures. Marinach-Patrice already in 2010 spiked blood culture bottles with 12 strains of six different *Candida* species and built up a reference database. Using this spectral database, they were able to correctly identify 36 *Candida* strains correctly in blood cultures [83]. In the same year, Lindsay Stevenson published a study with 212 positive blood culture broths. The overall accuracy for direct identification from positive bottles was 65% on the species level and 76% on the genus level [84]. It was shown that the number of bacteria necessary for identification was as low as 1×10^6 cfu. So it was higher than mass spectrometry-based identification from plate culture (5×10^5 cfu), but still about 3 logs less than conventional biochemical identification (5×10^8 cfu). Because an accuracy of 76% is not recommended for good routine identification protocols, the authors investigated the score value of the MS results. More than 95% of the isolates with a spectral score above 1.7 (Bruker recommends 2.0 for genus ID and 2.3 for species ID) had correct accordance with other ID methods. The authors concluded that a spectral score of 1.7 might be enough for direct identification from positive blood cultures. Still, one problem remained with septicemia diagnostics, as all eight *S. mitis* strains were misidentified as *S. pneumoniae*, a weakness of the spectrum discrimination (already discussed in the ID section). Even in 9 out of 10 positive

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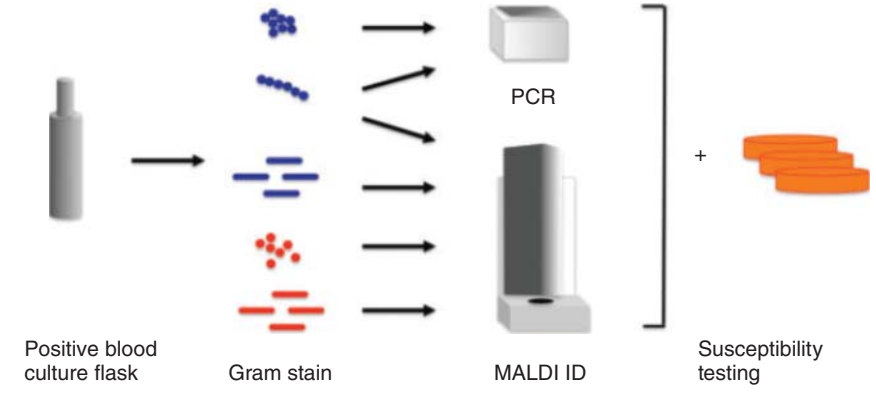
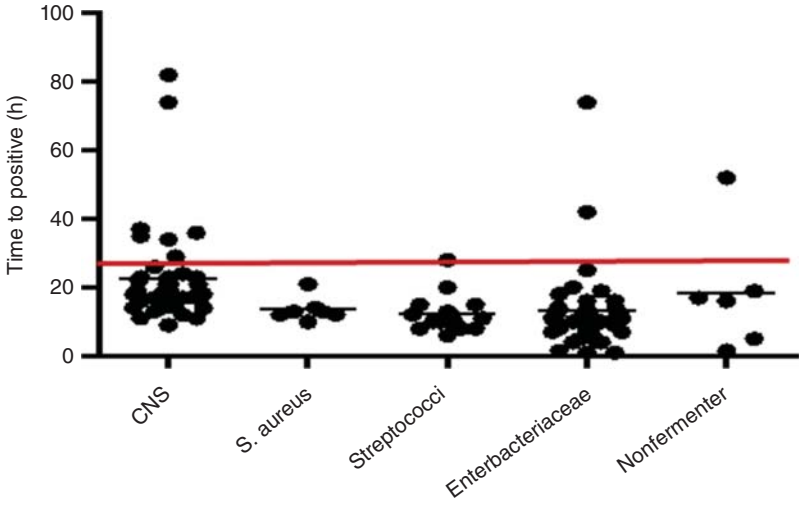
blood cultures with polymicrobic results, the identification of at least one microbe was possible. The authors concluded that the method provides a rapid, accurate, and definitive identification of bacteria within 1 h of detection in positive blood cultures with the caveat that the identification of *S. pneumoniae* would have to be confirmed by an alternative test. Much worse were the results published by Szabados and colleagues. They found an accuracy rate of 28% in a preliminary study with 50 positive blood culture bottles. A larger study setup confirmed the preliminary results when only 86 of 268 positive culture broths were correctly identified [85]. The main difference between Szabados' investigation and the work of La Scola and Stevenson was that the latter used the BD BACTEC blood culture system while Szabados used the BacT/ALERT system from Biomérieux. When the latter used the BACTEC system in direct comparison, the accuracy improved from 28 to 76%. The authors concluded that the charcoal, which was an ingredient of the BacT/ALERT system at that time (to bind, e.g., antibiotics in the blood culture broth), was responsible for the discrepancy.

After a number of study groups worldwide had shown that direct identification of microbes by mass spectrometry has acceptable accuracy, at least when using the Becton Dickinson BACTEC system, the hands-on time of the assay with several washing steps was close to an hour. As automated blood culture instruments deliver positive bottles for further processing as a 24/7 device, the procedure was still too laborious to be done for each single bottle. This changed when the first commercial assay for the identification, the Sepsityper™ kit from Bruker, became available. This test system requires only two centrifugation steps with volumes of 1 ml, and all centrifugation steps are performed in a single tube in the same centrifuge. It reduces hands-on preparation time from 30 to 50 min, as reported in the mentioned studies, to 5–10 min, which was now compatible with the workflow of a routine microbiology laboratory. It was first evaluated by Schubert's group in Munich, where more than 500 aerobic and anaerobic blood culture bottles were tested with the new kit [86]. In 87% of the samples, the identification was correct on a species level when a slightly modified score was used (spectral score above 1.7 and 3 consecutive hits in the result list). Even in mixed samples, at least one component could be identified in 18 of 27 broths. Only a few weeks later, Bode and colleagues confirmed the accuracy and the short performance time of the new assay. On a species level, 102 of 123 samples were correctly identified, resulting in an accuracy of 83% [87]. Fourteen of the 21 errors occurred in the group of coagulase-negative staphylococci. The clinical relevance of the exact species within the group of coagulase-negative staphs is very low; if these isolates were grouped as the *S. epidermidis* group, the accuracy increased to 94%. In the clinical setup of septicemia, the appropriate empiric therapy and, if necessary, a rapid adjustment of treatment is the key for the survival of the patients [18]. Therefore, the rapid MS identification was combined with a rapid PCR detection of the two most threatening resistance phenotypes in Gram-positive bacteria: methicillin resistance in *S. aureus* (mecA gene) [88] and vancomycin resistance in enterococci (*vanA* and *vanB* gene) [89]. Knowing the reason of the septicemia and the key resistance of



(a)



(b)

Figure 5.4 (a) Workflow for direct identification of positive blood cultures by mass spectrometry. (b) Time-to-positivity of blood cultures in different bacterial groups.

the Gram-positives, empiric antibiotic therapy could be adjusted very rapidly if one of the multiresistant Gram-positive threads occurs (Figure 5.4). About 75% of the most dangerous sepsis-inducing bacteria, such as enterobacteriaceae and *S. aureus*, resulted in positive blood cultures in <12 h. As the identification can be available in <15 min, and key resistance genes can be detected in <2 h, positive results of blood culture broths are now possible within an extended day shift of the lab including the time span for growth in the automated system. The first study in the United States was performed by Ledeboer's group in Wisconsin, analyzing 164 prospectively collected positive blood cultures representing 134 unique bacteremic episodes [90]. The MALDI Biotyper/Sepsityper identified 85.5% of bacterial isolates directly from positive monomicrobial blood cultures with 97.6% concordance to genus and 94.1% concordance to species with routine

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identification methods. Gram-negative isolates were more likely to produce acceptable confidence scores (97.8%). An important aspect was the median times to identification, which was 60 h shorter for Gram-positives (23 h instead of 83 h) and 17 h (34 h instead of 51 h) shorter for Gram-negatives. The time-to-result was reduced by 75 and 33%, respectively.

The enormous reduction in the time-to-result could lead to the conclusion that there must be an impact on patient's benefit in septicemia. Anne Vlek first raised this question and conducted a study comparing 89 bacteremic episodes with the rapid Sepsityper identification to 164 episodes with standard care. The time to identification went down from 45 to 16 h (reduction of 65%) and the time to the first adjustment of the antibiotic treatment was shortened from 24 h to less than 18 [91]. This resulted in an optimization of treatment within the first 24 h, which is crucial for the outcome of the septic patient, according to Kumar [18], in 40% of the patients (standard care only 29%). The proportion of septicemia patients receiving appropriate therapy was significantly higher with mass spectrometry identification (75%) compared to conventional diagnostics. Musser's group in Houston also analyzed the beneficial effects of the new technology for patients and the health system [92]. They found out that the integration of the rapid identification technique into an antimicrobial stewardship program with a rapid reporting system reduced the adjustment of therapy from 75 to 29 h (reduction >60%). The length of hospital stay of the patient after onset of the bloodstream infection dropped significantly from 10 to 8 days. The total hospital costs in the intervention group (using Sepsityper) dramatically reduced from $46.000 to $26.000 per septic episode. This proved that mass spectrometry-based identification of septic episodes not only has beneficial effects for the individual patient but also has ramifications for other areas of patient care.

Rapid pathogen identification using mass spectrometry can be used for additional clinical samples, not only blood culture broths. Ferreira used a centrifugation protocol similar to the earlier blood culture techniques to directly identify bacteria from urine of patients with urinary tract infections [93]. Two-hundred and twenty clinical samples with a significant number of bacteria to support the tentative diagnosis of UTI ($>10^5$ cfu/ml) were investigated without culture by direct MS identification, resulting in a correct species identification of 92% and an accuracy at the genus level of 93%. As the culture time was saved in this MS approach, the time-to-ID could be shortened from 16–24 to 1 h. In a consecutive study, the same group compared different protein extraction methods. The bacteria were pelleted from urine by centrifugation and either deposited directly on the MALDI target (intact cell method), while a protein extraction method was also performed (described in Section 6.2). Centrifugation of 4 ml urine resulted in 97% correct identifications with the intact cell method, which was comparable to the more laborious extraction method [94]. In a recent study, Burillo and colleagues modified the workflow and added the Gram stain to the mass spectrometry technique for direct urine identification. Their serial algorithm provided information within 1 h, which was consistent with 96.1% of urine samples from patients with suspected UTI [95]. Another group not only

centrifuged the urine but also used a diafiltration method to concentrate the bacteria for further identification with MALDI-ToF MS [96]. The turnaround time for the detection of microorganisms was 2–3 h instead of 24–48 h, and the sensitivity of the technique was 100%. Only the specificity was insufficient, with only 67%, due to a detection limit of 105–106 cfu per ml.

5.5 Antibiotic Susceptibility Testing Using Mass Spectrometry

The identification of microbes is not the only application for mass spectrometry in microbiology. After the substantial progress Hillenkamp and Tanaka achieved with the new technology, researchers relatively early started attempts to detect also resistance markers of bacteria by mass spectrometry. The field was fostered by an enormous increase in bacterial resistance not only in Europe but worldwide. Multiresistant phenotypes of bacteria can complicate the treatment of infections in critically ill patients and delay the start of appropriate and effective therapy [97]. Meanwhile, not only multiresistant but also pan-resistant strains, resistant to all available antibiotics, have been described [98, 99]. The multiresistant Gram-negative bugs are a more recent trend, while the resistant Gram-positive ones have already been known for decades. The increase of MRSA resulted, for example, in Portugal in a higher rate of *methicillin-resistant S. aureus* isolates in blood cultures than *methicillin-susceptible S. aureus* (MSSA). This development results in high in-hospital mortality, especially in the elderly [100]. Already in 2000, Gordon's group in Manchester typed 20 MRSA strains using intact cell mass spectrometry (ICMS) and defined specific peaks for MRSA [101]. In addition they detected peaks typical for MSSA and concluded that ICMS may have the potential for MRSA identification and typing, and may improve infection control measures. Two years later, Du and colleagues in Beijing published confirmatory data when they found that the spectral profiles of MSSA and MRSA differed greatly from each other [102]. They used a *nuc* and *mecA* gene-based PCR to control the results, and drew the conclusion that comparison of MALDI-TOF MS spectral profiles of microorganisms could serve as a simple and rapid method for bacterial identification and antibiotic susceptibility analysis. In this study, seven *mecA*-negative strains were misidentified as MRSA strains, which was explained by the authors as due to the heterogeneity of methicillin resistance in *S. aureus*. In the same year, Bernardo and colleagues confirmed the doubts that have been created by the misidentified strains when they stated that no uniform signature for MRSA could be detected in the spectra [103]. But this study demonstrated that MALDI-TOF MS is a powerful method for rapid identification of clonal strains of *S. aureus*, which might be useful for tracking nosocomial outbreaks of MRSA and for epidemiologic studies of infectious diseases in general. The lack of a uniform signature for MRSA versus MSSA was later substantiated by Szabados *et al*. They intensively analyzed an isogenic *SCCmec*-harboring parent and an *SCCmec*-lacking daughter strain, with the same genetic background,

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to unequivocally rule out strain-specific protein peaks, and could not show differences in the peak profile [104]. Majcherczyk not only focused on methicillin but also investigated teicoplanin resistance of *S. aureus* isolates. The MALDI-ToF MS was found to show a superior discriminatory power compared to PFGE or peptidoglycan muropeptide analysis for clonal strain analysis. Convincing test results in terms of teicoplanin resistance or susceptibility were not provided [105]. One study was published that demonstrated the ability of MALDI-ToF MS to distinguish between vancomycin-resistant enterococci and susceptible isolates. Griffin and colleagues analyzed 67 *vanB*-positive *E. faecium* strains and were able to predict susceptibility or resistance to vancomycin by the spectral peaks [106]. The sensitivity of the assay was 97% and the specificity was 98%. Up to now, this pattern analysis and profiles were only used in one laboratory. Therefore the possibility of effects of clonal strains is not ruled out completely yet.

As David Livermore stated in his remarkable review "Fourteen years in resistance," the situation of multiresistant Gram-positive cocci has improved, but that of Gram-negative rods has worsened over the last decade [97]. In recent years, especially, the percentage of carbapenem-resistant bacteria has increased at an alarming pace and become a major threat for patient survival. This development culminated in the discovery of the NDM-1 carbapenemase (New Delhi metallo-ß-lactamase), an enzyme degrading all ß-lactam antibiotics, not only in India and Pakistan but also in Europe [107]. Bonomo's group in Ohio was the first to investigate the stability of carbapenems against OXA-1 carbapenemases (Oxacillinase-1) using mass spectrometry. They used a quadrupole time-of-flight mass spectrometer (QMS) with a nanospray source to measure the binding between carbapenem antibiotics and bacterial carbapenemases to predict susceptibility and effectiveness [108]. But a QMS is not a standard instrument available in many labs, and binding affinity is not a direct marker for antibiotic degradation. Two groups in Europe developed a very similar assay at the same time, independently, using a simple biochemical calculation of the degradation process. When a ß-lactam ring in an antibiotic substrate is degraded by a ß-lactamase, the ring has to be hydrolyzed, so water is added to the ring (additional mass of 18 Da). In the next step (at least for carbapenemases), this unstable molecule is decarboxylated (means a mass subtraction of 44 Da). So the total mass of the inactivated ß-lactam antibiotic is 26 Da lower than that of the base product. This mass difference can be measured in a MALDI-ToF mass spectrometer. Burckhardt and colleagues used ertapenem as a test substrate, because it was known as the most sensitive carbapenem to detect resistant phenotypes and is one of the most stable carbapenems [109]. The ß-lactam was incubated with multiresistant Gram-negative rods harboring carbapenemase enzymes, such as NDM-1. After different time points, the bacteria were centrifuged and the supernatant (with the carbapenem) was spotted at a standard steel target and analyzed in a Bruker microflex™ mass spectrometer. Ertapenem with a molecular mass of 476 Da is applied to patients as a sodium salt. The mass spectrometer depicts not only the pure substance at 476 Da but also two sodium salts at 499 and 522 Da. After a short incubation of only 1 h, the three mass peaks

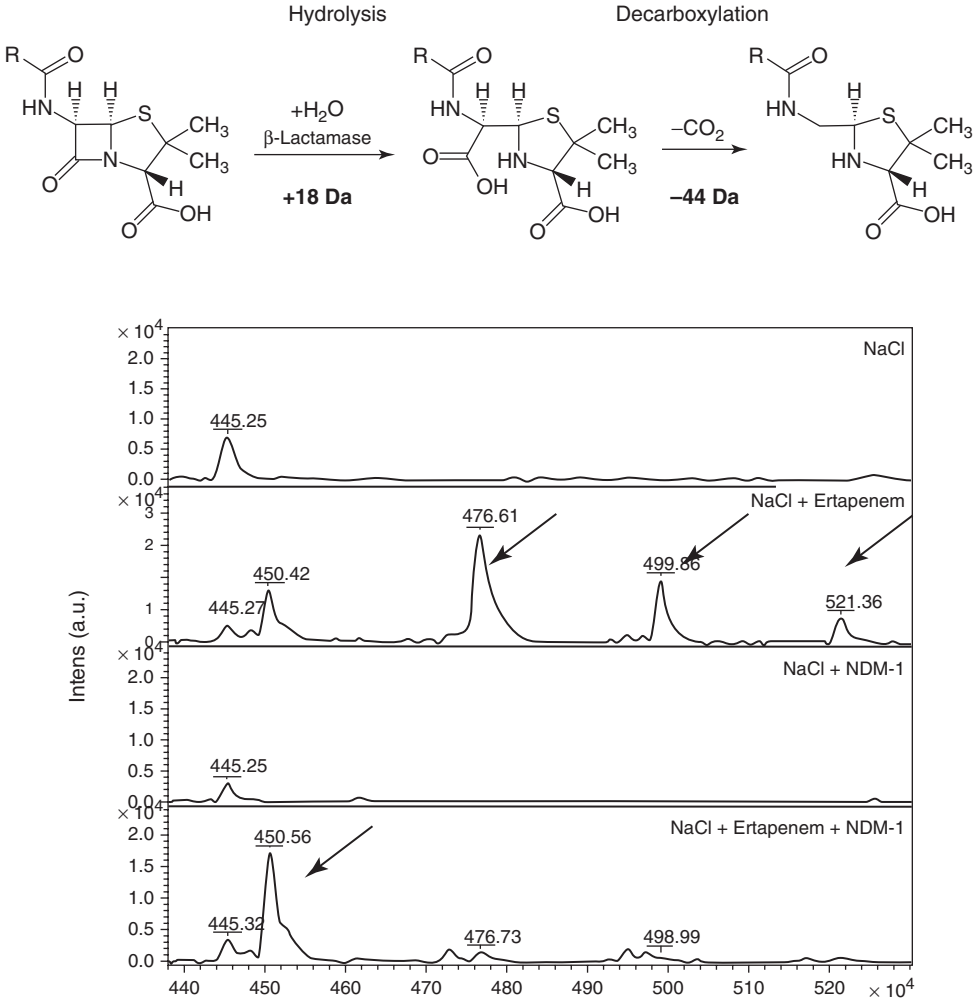

Figure 5.5 ß-Lactamase detection by MALDI-ToF.

of ertapenem are degraded, and a degradation peak at 450 Da (−26 Da) is visible (Figure 5.5). This assay delivers rapid results for a number of carbapenemases in different bacteria (enterobacteriaceae, Pseudomonas, and other Gram-negative non-fermentative rods). Hrabák and colleagues simultaneously investigated 124 strains, including 30 carbapenemase producers, using meropenem as a carbapenem target [110]. Both groups stated that the described method of carbapenem resistance detection has many advantages compared to agar-based methods or PCR. First, depending on the type of carbapenemase, results can be available as soon as 1 h after the start of incubation. This is especially useful in an outbreak situation where the carbapenemase is already identified. Second, this method is comparatively easy to perform. Only readily available reagents

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are used, and because MALDI-TOF MS is increasingly used in routine microbiology laboratories for the identification of bacterial strains, the hardware is already present in many laboratories. Third, the cost per determination is very low, less than €1 per reaction. Finally, we want to stress the universality of the method. The principle of degradation product monitoring can be applied to other enzymatic resistance mechanisms. A disadvantage of these studies is that both groups used only carbapenems as substrates and therefore detected only carbapenem resistance. A few months later, Sparbier and colleagues published a study detecting different ß-lactam molecules. They developed a method for the detection of ampicillin, piperacillin, cefotaxime, ceftazidime, ertapenem, and meropenem, and analyzed the different degradation products and sodium or potassium salt variants [111]. Though protocols for all groups of ß-lactam antibiotics were available now, a restriction of the new assays was that they can only detect degrading enzymes, but not other molecular mechanisms of bacterial resistance like target modifications, efflux pumps, or porin loss. Despite the fact that MALDI-ToF MS is a relevant tool for the detection of antibiotic resistance, the application cannot replace standard susceptibility testing because of these limitations [112].

Sparbier and Kostrzewa recently described a new assay overcoming these limitations. The idea was to grow intact microorganisms in drug-containing, stable, isotope-labeled media and to acquire mass spectra from these microorganisms. These spectra were compared with mass spectra of the intact microorganism grown in nonlabeled media without the drug present. Drug resistance was detected by characteristic mass shifts of one or more biomarker peaks. The method was based on a publication by Demirev and colleagues, who used to commercially available media in which almost all of the C atoms are ^{13}C. The growth of bacteria in a medium in the presence of an antibiotic is the evidence of its resistance against this drug. This evidence is given by the peak shifts caused by the ^{13}C incorporation into the biomarkers [113]. With this method, resistance detection by MALDI-ToF mass spectrometry can be expanded also to antibiotics that show no enzymatic degradation. A disadvantage is that the completely labeled medium is costly. Sparbier *et al*. grew their bacteria in normal medium where only one lysine as a single amino acid was labeled with $^{13}C_6$ $^{15}N_2$-L-Lysine, which is more cost effective. In this supplemented medium, they grew *Klebsiella pneumoniae* strains with or without meropenem. After 2 h of incubation, the spectrum acquired from the resistant bacterium in the labeled medium with the antibiotic was nearly identical to the spectrum from the organism grown in the labeled medium without meropenem. In contrast, as the growth, and thereby protein synthesis, of the susceptible strain is inhibited, its spectrum after incubation in the labeled and antibiotic-containing medium is still similar to the "normal" spectrum [114]. Sparbier used the new approach to distinguish methicillin-susceptible *S. aureus* strains from resistant ones (MRSA). Oxacillin and cefoxitin were used to analyze 20 *S. aureus* strains on their resistance phenotype by mass spectrometry, and the result were compared with those from conventional culture-based methods and PCR [115]. Nineteen

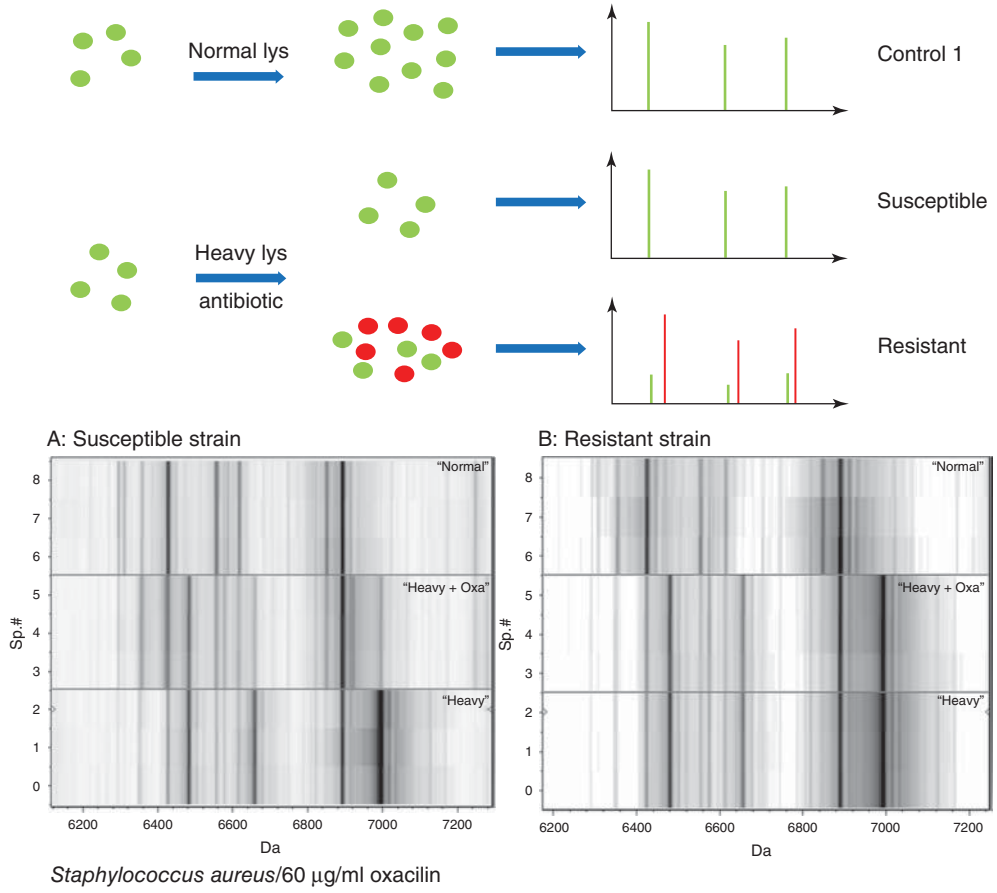

Figure 5.6 MS resist assay using heavy, stable isotopes.

out of 20 strains showed correct results within 3 h. The strains were inoculated in three different broths: one with nonlabeled medium without antibiotics, one with antibiotics without heavy lysine, and one with antibiotics and heavy lysine containing medium (MS resist, Figure 5.6). After the incubation period, the mass spectrum of susceptible strains showed high similarity between the unlabeled medium and the labeled medium with antibiotic. As the strains cannot grow in the presence of oxacillin, no main changes in the spectra occurred. MRSA strains showed no growth inhibition with oxacillin or cefoxitin, so the peaks were similar between the heavy isotope medium and the heavy isotope antibiotic approach. The time-to-result for a conventional antibiotic susceptibility test is at least 5–6 h, so it can be halved with the new assay. As already mentioned, rapid testing is the precondition for appropriate therapy and effective antibiotic stewardship [92]. Trecharichi and colleagues recently emphasized the important role of *P. aeruginosa*, especially multi-resistant strains, in neutropenic patients

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[116]. *Pseudomonas* is the second most common isolated microbe in these patients and linked to a high mortality especially when bloodstream infections occur. Jung *et al.* investigated *P. aeruginosa* resistance using the new and rapid mass spectrometry technique based on stable isotopes [117]. They were able to test 30 different *Pseudomonas* strains with the three different key antibiotics: ciprofloxacin, tobramycin, and meropenem. All of the strains showed good correspondence between the susceptibility interpretation of conventional test methods and the MS resist protocol. Therefore, this new assay seems to be a promising tool for a multiplicity of antibiotic substances, reporting rapid and reliable results.

The detection of antibiotic resistances in bacteria is a hallmark of clinical microbiological diagnostics. Though time consuming, culture-based methods for antibiotic resistance testing are still the gold standard in diagnostic laboratories around the world. The introduction of MALDI-TOF MS in medical microbiology has already tremendously changed the way of identifying bacteria and fungi. Now, the first steps are being taken to exploit MALDI-TOF MS for the fast detection of antibiotic resistances. These results are very promising and will encourage a further agile development of this technique.

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6
IR and Raman Spectroscopy for Pathogen Detection

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6.1
Introduction

The presence of bacteria, the amount of cells, or even the identification of bacterial species or strains is necessary for various fields [1]. Depending on the purpose of the application, it is often only necessary to know the bacterial load. However, for medical purposes more information is required. The exact identification of bacteria is necessary in order to provide the appropriate therapy and to prevent antibiotic resistance.

For the conventional microbiological identification, the first step is always a separation of the bacteria followed by a visual inspection of the colonies as well as a microscopic evaluation of the cell morphology and a Gram typing. The next step is to test their ability to grow in various media under different conditions according to their biochemical properties [2]. This method is especially suited for parallel identification. Nevertheless, since it is based on bacterial growth, it takes at least some hours until the results are available, but depending on the type of bacteria normally much longer [3–5]. Alternative identification approaches are polymerase chain reaction (PCR)-type identification methods, fluorescence spectroscopy, flow cytometry, chromatography, or chemiluminescence techniques, which often require pure samples [2, 6, 7].

In addition to these techniques, vibrational spectroscopy such as IR absorption and Raman spectroscopy can enable the detection of biochemical information of the cells normally without the need for destruction of the sample. During vibrational spectroscopy, the vibrational and rotational modes of molecules are altered by delivering energy quanta via electromagnetic irradiation. Since each vibrational mode requires a specific amount of energy to be excited, this results in energy uptake at distinct frequencies corresponding to the specific vibrations in a particular molecule or chemical group. Since IR and Raman spectroscopy are based on absorption or scattering processes, respectively, the type of molecules that can be observed with these methods are different. In IR absorption spectroscopy, a change of the dipole moment during the vibration is necessary. Therefore, polar chemical groups such as, for example, C=O double bonds or amide

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vibrations, show very intense signals. In contrast, in Raman spectra signals with C=C double bonds or aromatic systems can be observed easily. The combination of all vibrational signals is specific for the respective molecule, which is why vibrational spectroscopy is frequently referred to as "molecular fingerprinting". The "optical fingerprint" of each substance allows the identification of different substances inside the cells. Since the biochemical composition of bacteria varies between species, serotypes, and strains, bacteria of the same taxon have a unique and specific combination of spectral features; thus, microorganisms can be identified from their vibrational spectra [8].

FT-IR (Fourier transform infrared) spectroscopy on bacteria was first introduced by Naumann *et al*. in 1991 [9]. The combination of especially FT-IR spectroscopy with distinct chemometric approaches has become a widely used method for the identification of bacteria and yeasts [10]. For IR absorption spectroscopy, normally dried bacterial films on KRS-5 plates (Figure 6.1b-i) or ZnS are used. An alternative approach is the use of different Raman spectroscopic techniques. Here, the need for sample drying is not compulsory, because the Raman spectrum

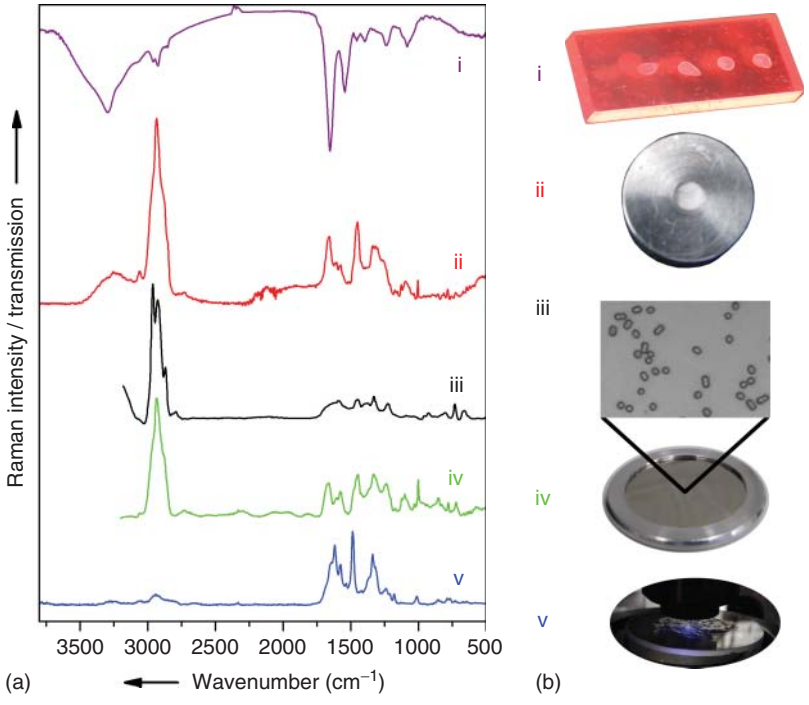

Figure 6.1 Vibrational spectra of *E. coli* using different vibrational techniques. Panel (a) shows the spectra measured with different excitation wavelengths. In (b) images of typical samples are depicted. i: IR absorption spectrum (bulk); ii: FT-Raman spectrum (excited with 1024 nm, bulk); iii: SERS spectrum (excited with 532 nm in microfluidic device, bulk); iv: Vis-Raman spectrum (excited with 532 nm, mean of single cells); and v: UV-resonance Raman spectrum (excited with 244 nm, bulk).

of water is not intense compared to the IR spectrum. Therefore, with Raman spectroscopy it is even possible to measure living cells. Since the first measurements of bacteria [11], the topic of identification of microorganisms by means of Raman spectroscopy has gained increasing interest as reflected by the increasing number of reports on the subject. Nevertheless, most Raman measurements were performed on solid fused-silica plates (Figure 6.1b-v) or on CaF_2 substrates, with special sample holders for dried bacteria samples (Figure 6.1b-ii), or a special Ni foil (Figure 6.1b-iv).

In Figure 6.1, one strain of *Escherichia coli* was investigated with IR spectroscopy (spectrum a-i) as well as Raman spectroscopy by applying different laser wavelengths for excitation. The use of excitation wavelengths in the visible or near-infrared (NIR) region (spectra a-ii and a-iv in Figure 6.1) normally leads to Raman spectra that monitor the whole cell chemistry. Therefore, this type of classification is called *phenotypic*. In contrast, the application of UV light (spectrum a-v in Figure 6.1) enables the selective enhancement of macromolecules such as DNA or proteins. Since these molecules are very specific as taxonomic markers, this makes UV-resonance Raman (UVRR) spectroscopy especially suitable for the classification and identification of microorganisms. Adding metallic nanoparticles (NPs) to the sample changes the spectrum in an entirely different way (spectrum a-iii in Figure 6.1), now displaying mostly signals of compounds that are in close proximity to the NPs. Therefore, by carefully choosing the spectrum acquisition parameters, vibrational spectroscopy can provide detailed insight into very different parts of microorganisms.

The combination of a Raman setup with a conventional microscope allows spatial investigation of samples in the submicrometer range. In combination with visible excitation wavelengths, this method enables the observation of single bacterial cells (spectrum a-iv in Figure 6.1).

Since the IR and Raman spectra of different species or even strains are very similar, statistical methods are necessary to differentiate between the analyzed classes. To this end, first, a database for a specific application has to be established (normally on cultivated bacterial samples) and the spectra from this database are classified by an appropriate chemometric model. The quality of this classification can then be validated by assigning independent test batches of the analyzed bacteria. The final test of the model is the identification of real-world samples using the database and the established statistical model.

In this chapter, we give an overview over the different vibrational spectroscopic approaches in the field of bacterial identification. Therefore, we focus in the first part on IR and Raman spectroscopy of bulk samples. Because of the necessary cultivation step for generating enough biomass for the analysis, the methods can easily be standardized. In the second part, we discuss some approaches for single-cell analysis. Here, no standardization is possible. Therefore, both the cell-to-cell variation and the phenotypic variation due to different growth conditions have to be taken into account.

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6.2
Bulk Analysis

The investigation of microorganisms with spectroscopic techniques is frequently carried out on bulk materials. To obtain this, the samples have to be cultivated. This offers the opportunity to standardize the growth conditions in order to generate uniform samples. Also, if a cluster of bacteria is to be measured, all metabolic states that the bacteria may adopt are measured simultaneously and the resulting spectrum represents a robust average of the sample.

6.2.1
IR and NIR Absorption Spectroscopy

The infrared region (14 000 to 10 cm^{-1}) can be divided into three regions – near-IR, mid-IR, and far-IR. The far-IR and near-IR regions are usually not studied, as they contain overtones and combination vibrations, which are hard to study and interpret. The most useful vibrational frequencies are present in the mid-IR (4000 to 400 cm^{-1}) region, as spectral features of macromolecules (proteins, lipids, carbohydrates, nucleic acids, etc.) in bacteria can be observed there. Mid-IR spectra can further be divided into five major regions, which are shown in Figure 6.2. These regions are suggested to be used for the identification of bacteria. In region I (3000 – 2800 cm^{-1}) the absorption of aliphatic C–H stretching modes are found and this region is frequently correlated with fatty

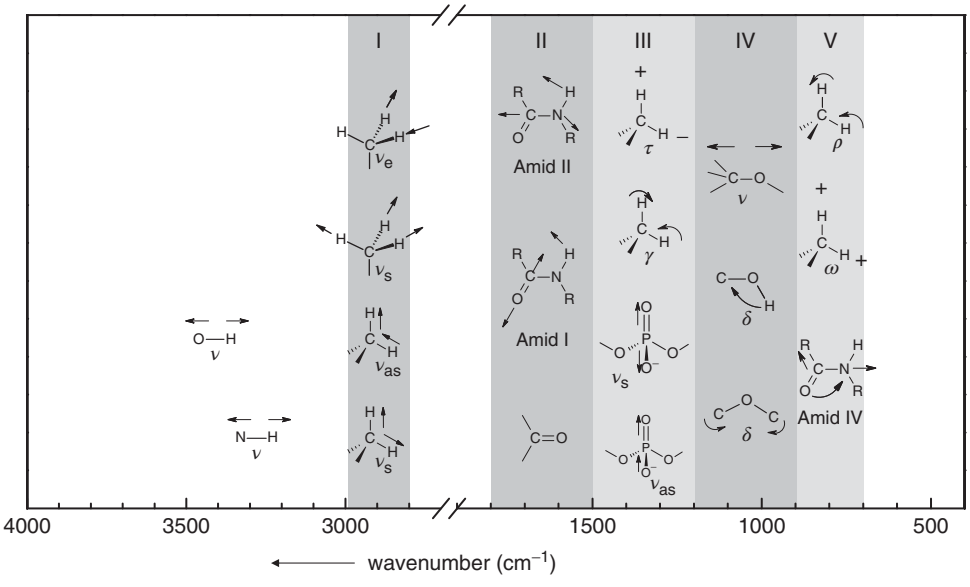

Figure 6.2 The five regions of interest for IR and Raman spectroscopy. Important characteristic vibrations found in biological molecules are shown schematically for each region.

acids. Region II (1800 to 1500 cm^{-1}) includes the carbonyl stretching vibration of lipids at 1740 cm^{-1} as well as the amide I and amide II bands of proteins and peptides at roughly 1650 and 1550 cm^{-1}, respectively. The amide bands can be used to deduce information about the structure of proteins (α-helix, β-sheet, random coil conformation) [12, 13]. Region III (1500 to 1200 cm^{-1}) shows a combination of fatty acid bending vibrations, protein signals, and vibrations from phosphate-bearing cellular components such as phospholipids. Region IV (1200 to 900 cm^{-1}) provides information about the structure of polysaccharides, and in region V (900 to 700 cm^{-1}), the so-called fingerprint region, rather weak but unique absorbances are found, which are characteristic for specific bacteria. Especially, regions I and II are the workhorses for routine bacterial identification. However, the other regions often help to better understand minor variations in structure and composition of the bacteria. Finally, discriminating bacteria on the strain level heavily relies on the fingerprint region. The first suggestions to analyze biological material by means of infrared spectroscopy were made in the early decades of the twentieth century [14].

Although the benefits of IR spectroscopy – above all the potential for reagentless analyses – were immediately recognized, first attempts to identify bacteria via molecular spectroscopy in the 1950s had limited success. The technique only became useful after the advent of the FT-IR spectroscopy and the utilization of chemometric tools in the late 1980s and 1990s. Especially, the inspiring works of Naumann and coworkers reintroduced FT-IR methods in combination with chemometric tools for *in situ* analyses of bacterial cells, which resulted in a boom in scientific publications over the past 20 years [8, 9, 15]. Since then, FT-IR spectroscopy has been successfully used to study, among others, biofilm formation, cellular components in bacteria, microbial response to stress, injury, and inactivation, as well as the development of antibiotic resistance [16–18]. Emphasis was also placed on the detection, discrimination, classification, and identification of bacteria – especially foodborne pathogens – such as *Bacillus* [19, 20], *Brucella* [21], cyanobacteria [22], *E. coli* [23, 24], lactobacilli [25], *Listeria* [26, 27], *Mycobacteria* [28], *Streptococcus* [29], *Salmonella* [30, 31], *Staphylococcus* [32, 33], and *Yersinia* [34, 35]. Also nosocomial pathogens, such as the members of the *Burkholderia cepacia* complex [36–38] possible warfare agents [39], and even fungi [40] were investigated. Thereby, samples in different conditions (liquid, suspended, powdered, or dehydrated) could be measured thanks to a variety of sampling methods and techniques available to record the spectra. The most common methods of measurement with regard to the characterization of bacteria are transmittance, diffuse reflectance, attenuated total reflectance (ATR), and microspectroscopy. The utilization of chemometrics or multivariate statistical analyses for high-dimensional spectral analyses allows the extraction of qualitative and quantitative information from numerous spectra simultaneously, which is impossible by a mere "stare and compare" interpretation. The achieved levels of taxonomic resolution cover genera, species, subspecies, and infrasubspecific ranks, depending on the task at hand. For example, the group of Rau investigated several *Yersinia*

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species by means of transmission FT-IR spectra [34, 35]. Using a hierarchical artificial neural network classification scheme, they first distinguished *Yersinia* from more than 1000 other Gram-negative isolates on the genus level. In a subsequent step of species identification, *Yersinia ruckeri*, the causative agent of the important bacterial fish disease, enteric redmouth disease, was identified out of 200 *Yersinia* strains. Forty-nine *Y. ruckeri* isolates were used for validation in this study, some of which were cultured field isolates from diseased salmon fish from several fish farms in Germany. Correct identification of 97.4% of *Y. ruckeri* was achieved [35]. In a similar study, *Yersinia enterocolitica* was successfully distinguished from other *Yersinia* species. But also the biotype and serotype of the *Y. enterocolitica* isolates were correctly determined with accuracies of 98.3 and 95.5%, respectively [34].

Subtyping of species is of particular importance, because different subtypes can have different virulence and pathogenicity. In several studies, different groups have proven that FT-IR spectroscopy in combination with chemometric methods is a versatile tool also for subtyping of bacteria. For example, Davis and coworkers subtyped 30 closely related strains of *E. coli* O157:H7, which cause water- and foodborne human illnesses worldwide [41]. Subtyping of *E. coli* O157:H7 provides information about the genetic, phenotypic, and virulence attributes of the bacteria, which is vital for pathogenesis evaluation and an early reaction after a possible outbreak. The developed FT-IR/chemometric approach was shown to be suitable for rapid ($\leq$16 h) subtyping of *E. coli* O157:H7 with a comparable accuracy to MLVA (multilocus variable number tandem repeat analysis) typing. Also, a spectral relatedness between different outbreak strains was found, and a classification based on the type of Shiga toxins was achieved with an accuracy of 97%. Vaz *et al*. effectively used FT-IR transmission spectroscopy in combination with PLS-DA (partial least square discriminant analysis) for subtyping of *Staphylococcus pneumoniae*, a notorious cause of community-acquired pneumonia. They successfully determined the serotype of 69 invasive, encapsulated *S. pneumoniae* strains from pure culture. For strains of an independent set of test strains, the correct serogroup was predicted with 100% accuracy [29]. A simultaneous typing of species and strains of lactic acid bacteria (specifically probiotic vs environmental *Enterococcus faecium*) by means of FTIR spectroscopy and artificial neural networks was presented in a study of Wenning *et al*. [42]. Validation experiments achieved accuracies of 93.2% at the species level and 97.1% at the strain level. This is good news for the food industry, as lactic acid bacteria are highly relevant for fermented food and beverages, and fast and easy species or strain typing is necessary for quality control. Also, foodborne pathogens such as *Listeria monocytogenes* or *Salmonella enterica* were successfully subtyped to the haplotype and serotype using FT-IR [27, 30].

However, it should be mentioned that a careful standardization of growth conditions, sample preparation, and spectral measurements were the cornerstones to guarantee a sufficiently high reproducibility in the above-mentioned publications. While FT-IR spectroscopy is fast (typically 10 s to 1 min per sample), the approaches for microbial sample preparation can be time consuming because lengthy plate cultures or fluid cultures in shake flasks are required for growth of

the organism. Micro-cultivation and the application of FT-IR microscopy might increase the speed of analysis [43, 44]. Also, the rather high limit of detection (about 10^3 cfu/ml) compared with other techniques, such as immunochemical and nucleic acid analysis, for the identification of bacteria-specific features by IR spectroscopy is an issue when bacterial batches cultured in broth or on agar are not practicable [16]. Especially in the case of low analyte concentrations, the strong IR absorption of water poses a problem, but it can be minimized to some degree by partially dehydrating the cells prior to the measurements [45]. A further option to concentrate bacteria and remove as much water or media components as possible from the samples is the collection of bacteria on filter material or with the help of magnetic NPs (immunomagnetic separation; IMS). Recent publications took advantage of these harvesting techniques to detect, differentiate, or enumerate targeted bacteria from cultivation media or complex matrices, especially foodstuffs such as fruit juice, milk, or chicken breast [31, 46–48]. For instance, *E. coli* O157:H7 was isolated from artificially contaminated ground beef by filtration or IMS to eliminate spectral interferences due to the meat. A detection limit of 10^5 cfu/g for both methods was achieved, as well as a differentiation of three outbreak strains of *E. coli* O157:H7 from an apathogenic control strain. Furthermore, partial least square (PLS) models with cross validation made it possible to establish a relationship between plate counts and FT-IR spectra and to quantify different ratios of live and heat-treated (dead) cells of *E. coli* O157:H7. Times to detection accounted for 1 and 4 h for filtration-FT-IR and IMS-FT-IR, respectively, when a bacterial load of 10^5 cfu/g was given. However, the proof that detection and identification of *E. coli* isolated from real samples is possible has not yet been provided [23].

A total avoidance of enrichment steps is possible in case of direct FT-IR and FT-NIR methods. For example, pathogens were measured *in situ* in milk, pulp juice, or on chicken filets [33, 49, 50]. Also, results about a comparison of Raman and FT-IR spectroscopy for the prediction of meat spoilage were recently published: minced beef samples were stored under different packing conditions. Time series spectroscopic, microbiological, and sensory analysis data were obtained from these samples and models predictive of the microbial load and sensory assessment were calculated by a collection of multivariate statistical methods. FT-IR models performed slightly better in predicting the microbial counts (subdivided into total viable counts, *Brochothrix thermosphacta*, *Enterobacteriaceae*, lactic acid bacteria, pseudomonads, yeasts, and molds) than their Raman counterparts. Still, it was concluded that both Raman and FT-IR spectroscopy can be used reliably and accurately to rapidly assess meat spoilage by creating larger databases and by choosing appropriate prediction models [51].

In conclusion, for bulk samples, IR spectroscopy has been proven to be well applicable for discriminating bacteria at different taxonomic levels under various conditions if a rigid standardization of all steps of sample preparation is executed. The differentiation capability of the method is promising; however, an application toward identification of independent validation samples, or even real samples,

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is still to be made in most of the works. Nowadays, commercial instruments for microbial identification by FT-IR spectroscopy are available.

6.2.2 Raman Spectroscopy with Excitation in the NIR Region

Similar to IR spectroscopy, Raman spectroscopy with excitation wavelengths in the near infrared region (785–1064 nm) results in phenotypic spectra that contain information of the whole cell components. In contrast to IR spectroscopy, Raman spectroscopy offers the opportunity to investigate wet samples or bacteria directly in an aqueous environment. This allows the investigation of living microorganisms. The first approach to characterize bacteria by means of FT-Raman spectroscopy was made by Naumann *et al*. in 1995 [15]. The Raman spectra of dried and ground bacteria were compared with their FT-IR spectra. Both the IR and Raman spectra were then used for distinguishing Gram-negative and Gram-positive bacteria. Five years later, Maquelin *et al*. [52] applied a confocal micro-Raman setup with an excitation line of 830 nm to investigate clinically relevant microorganisms. In this study, two sample preparation methods were compared. The quality of Raman spectra from bacterial smears were compared to that of spectra measured directly on microcolonies on a solid culture medium. With this technique, the bacteria could be identified with a minimal cultivation time of 6 h.

Since the measuring position on a microcolony is not defined, several studies have been dedicated to investigate the spatial heterogeneity of microcolonies. Choo-Smith *et al*. [53] analyzed the heterogeneity of microbial colonies in axial and lateral directions. They found significant variations in the Raman spectra of older colonies with respect to the measuring position. These variations were assigned to an increase in glycogen content in the surface layer of the colony and an increase in the amount of RNA in the deeper layer. In addition, the carotenoid content of pigmented bacteria like *Staphylococcus aureus* was also found to be spatially dependent. Goodwin *et al*. [54] analyzed the distribution of carotenoids in small colonies of *Halobacterium salinarium* with an excitation wavelength of 633 nm and observed concentric rings of different carotenoid concentrations.

Besides the spatial heterogeneity of colonies, also the cultivation conditions influence the molecular composition of bacterial cells and therefore their vibrational spectra. Hutsebaut *et al*. [55] investigated the influence of different nutrition conditions on the quality of the Raman spectra. For this study, various strains of three *Bacillus* species were cultivated with different nutrition media, temperature, and cultivation times. The influence of these parameters on the recognition rate of the Raman spectra shows that standardized cultivation conditions are necessary for a reliable identification of bulk samples.

Despite these restrictions, Raman spectroscopy has been used in various studies for the classification of medically relevant bacterial species or even strains. Maquelin *et al*. [56] investigated 25 strains of *Acinetobacter* from different hospital outbreaks. Raman spectroscopic grouping of the strains by HCA

(hierarchical cluster analysis) was found to be in good accordance with grouping results obtained by a DNA fingerprinting technique. In another study, Kirschner *et al.* [57] demonstrated that vibrational spectroscopic classification of *Enterococcus* species correlated well with data from 16S RNA analysis. Some strains, however, were misclassified by both FT-IR and Raman classifications. Further, 16S RNA sequencing of these strains led to their taxonomic reclassification. This shows that vibrational spectroscopy can even help to improve taxonomic classification of bacteria.

It should be noted that Raman spectroscopy is also applicable to investigate bacterial mixtures qualitatively and quantitatively. For example, the group of Berger carried out a series of experiments on oral microflora bacteria. In a preliminary study, they measured four different *Streptococcus* species directly on agar plates [58]. After correcting the Raman spectra for the agar contribution, the applied HCA clustered the spectra according to their species identity. In further experiments, the relative concentrations of *Streptococcus mutans* and *Streptococcus sanguinis* were determined successfully from mixtures of the two species [59]. In a follow-up study, three *Streptococcus* species were used to create an artificial oral plaque. In these ternary mixtures, the concentration of *S. mutans* was predicted using PLS analysis [60]. To discriminate between *S. sanguinis* and *S. mutans* in biofilms, Beier *et al.* [61] constructed a prediction model with the spectra from pure biofilms of the two species. Pure biofilms of the two species grown at a later date served as validation data and were identified with 96% accuracy. A second validation dataset was obtained from artificial mixtures of the two species. In Figure 6.3, the white light image of the pseudo-mixed biofilm and the measurement positions are shown. With a spatial resolution of approximately 2 µm, a classification accuracy of 97% was obtained for the mixed sample.

Also in other aspects of hygiene, possible bacterial contaminations are of high interest. Yang and Irudayaraj [62] successfully used FT-Raman spectroscopy to

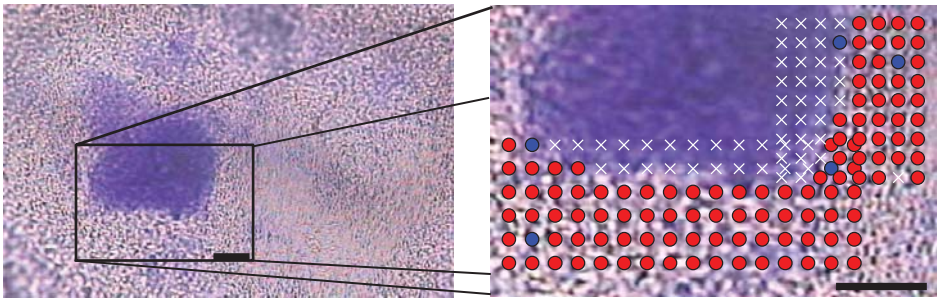

Figure 6.3 White-light transmission image of the *S. sanguinis* stained with crystal violet (darker regions) and unstained *S. mutans* (lighter regions) in a pseudo-mixed biofilm (a) with a close-up of the scanned region (b). Assignments in the scan: red circles, *S. mutans*; blue circles, *S. sanguinis*; ×, spectral outlier (e.g., due to the stain). Circle sizes represent laser focus (bar length = 10 µm). The figure is reproduced from Ref. [61] with permission of A. J. Berger and SPIE.

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discriminate bacteria on food surfaces. For this investigation, bacterial smears were brought onto apple surfaces and were measured directly. This method allowed the differentiation between pathogenic and nonpathogenic *E. coli* strains. Furthermore, Rygula *et al*. used FT-Raman spectroscopy to identify *E. coli* on the surface of cotton and polyester textiles. With this approach, it was possible to analyze the influence of microorganisms on different textiles and to discriminate between infected and clean materials as well as between previously washed and unwashed textiles [63].

Beside bacteria, also pathogenic yeasts have been the subject of various studies. For example, Maquelin *et al*. [64] differentiated between various *Candida* species prepared from blood cultures by means of sequential identification. For the evaluation of the method, samples from several patients were used and identified correctly [65]. Even samples from intensive care units were identified by means of Raman spectroscopy, showing good accordance with conventional identification techniques [66].

Apart from classification and identification, also a deeper insight into the chemical composition as well as the changes during growth are of interest and may be investigated with Raman spectroscopy. De Gelder *et al*. [67] applied two-dimensional (2D) correlation Raman spectroscopy for the determination of bacterial chemical components. For this study, the bacteria were harvested at different times during cultivation. The change in the relative concentration of different amino acids, nucleic acids, fatty acids, and components of the main metabolic pathway such as coenzyme A could be calculated. In another study, Mello *et al*. [68] used 2D correlation spectroscopy from samples with increased bacterial concentrations in order to obtain more detailed Raman information of the amide I band. Both the synchronous and the asynchronous plots for three species of different genera causing gastroenteritis could be used to differentiate between the bacteria.

A very interesting approach to obtain the cell numbers required for NIR Raman spectroscopy was presented by Schröder *et al*. in 2013 [69]. Instead of cultivating the bacteria to increase their numbers, they captured the bacteria from a fluid using a dielectrophoresis setup and subsequently measured Raman spectra from the captured bacteria. This powerful combination may enable the direct investigation of bacteria from body fluids such as urine, cerebrospinal liquid, or ascites without any cultivation.

Over the past years, Raman spectroscopy with excitation in the NIR region has been proven to be a useful tool for discriminating bacteria from their characteristic spectral patterns under different conditions. To obtain sufficient bulk material, samples are frequently cultivated. This allows the bacteria to be grown under standardized conditions, which increases the spectral reproducibility and results in more reliable classification and identification datasets. The combination of Raman spectroscopy with excitation in the NIR region with new methods of concentration and isolation can pave the way for the investigation of samples without cultivation, which also allows for easier analysis of bacteria that are difficult to cultivate.

It should also be noted that, by using excitation wavelengths in the NIR region, the problem of sample fluorescence, which frequently occurs when using visible excitation wavelengths, can be minimized or avoided entirely. Furthermore, the phenotypic information of a sample is not covered by chromophores that may be present, since chromophores do not absorb NIR radiation. However, the presence of compounds that show absorption at the excitation wavelength can be exploited via resonance Raman (RR) spectroscopy.

6.2.3 Resonance Raman Spectroscopy

In contrast to conventional Raman spectroscopy, RR spectroscopy allows a wavelength-dependent selective enhancement of molecular vibrations close to an electronic transition.

In biological samples, electronic transitions usually are absent in the NIR and IR wavelength range. However, chromophores may be present that can have strong absorption bands in the visible wavelength range. Figure 6.4 shows the absorption spectra of β-carotene (red) and cytochrome c (green) with electronic absorption maxima between 350 and 500 nm. Other molecules such as lysozyme (purple spectrum in Figure 6.4) show only an absorption in the UV range. Proteins and DNA absorb in the UV range because of the aromatic rings in their structures. These electronic absorptions are exploited in RR spectroscopy. Choosing excitation wavelengths in or close to the maximum of an electronic absorption enhances the Raman signal by a factor of up to 10^6. Therefore, this technique enables the detection of molecules with low concentrations down to 10^{-6} M inside a cell.

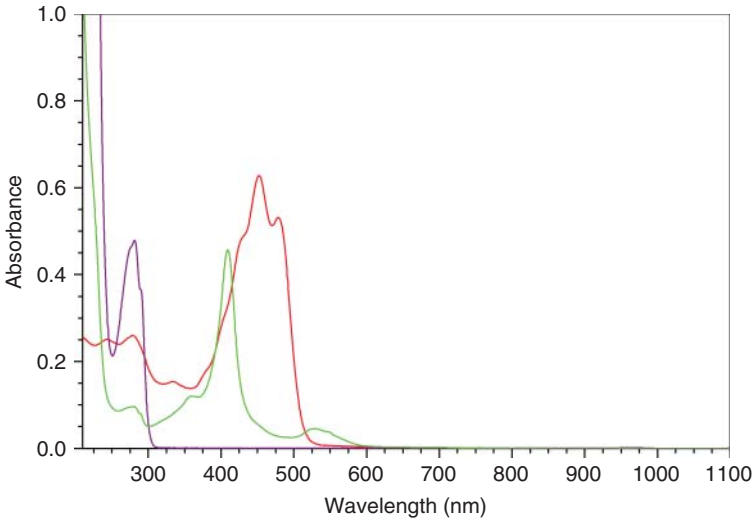

Figure 6.4 UV–vis absorption spectra of ß-carotene (red, ethanolic solution), cytochrome c (green, aqueous solution), and lysozyme (purple, aqueous solution). Note that in the NIR region there is no absorption from any of the compounds.

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6.3
Excitation with Visible Wavelengths

Inside complex biochemical structures, such as bacteria, the resonance effect frequently occurs because of chromophores such as carotenoids, cytochromes, or chlorophyll. Furthermore, some proteins, such as bacteriorhodopsin, which is used by some archaea as proton pump, show an enhanced Raman signal when excitation light in a visible wavelength range is used.

In Figure 6.5, the RR spectra of three typical chromophores measured with 532 nm laser excitation wavelength are shown.

Cytochrome b_{588} (spectrum a in Figure 6.5) shows intense Raman peaks at 745 cm^{-1} (ring breathing vibration of purine), 1125 cm^{-1} (C–N vibration), 1303 cm^{-1} (C–H deformation vibration), and 1580 cm^{-1} (asymmetric C–C vibration) [70]. β-Carotene (Figure 6.5, spectrum b) appears in a variety of organisms from bacteria and plants to animals, where the molecule is mainly involved in the process of photosynthesis because of its capability to absorb light in the visible wavelength range around 500 nm [71]. Its RR spectrum is dominated by two prominent bands at 1153 and 1515 cm^{-1} assignable to the C–C stretching and C=C stretching vibration, respectively [72]. Spectrum c in Figure 6.5 depicts

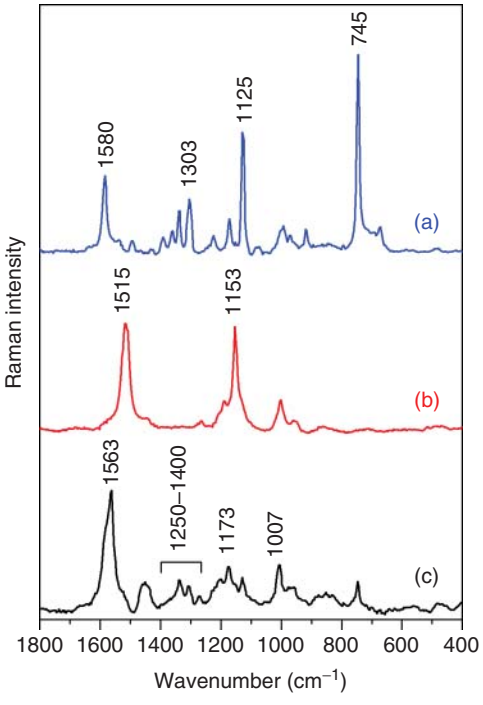

Figure 6.5 Resonance Raman spectra of cytochrome b_{588} (a), β-carotene (b), and bacteriorhodopsin (c) measured at 532 nm laser excitation wavelength.

a Raman spectrum of the carotenoprotein bacteriorhodopsin. In comparison to those of free carotenoids, the spectrum is more complex. Although signals of protein components could be expected, only carotenoid bands are enhanced by the resonance effect (Figure 6.4), whereas protein signals are overlapped and therefore hardly observable. The spectrum is influenced by mainly four vibrational modes: the C=CH out-of-plane wagging mode at 1007 cm^{-1}, the C–C stretching vibration at 1173 cm^{-1}, the C=CH in-plane rocking vibration in the range of 1250–1400 cm^{-1}, and C=C stretching vibration in the most prominent band region at 1563 cm^{-1} [73]. Observation, differentiation, and even identification of such components are always of interest, since they help to explain the cell physiology and to classify cells.

The first RR spectra were recorded from chromobacteria with an excitation wavelength of 488 nm. Howard *et al*. recorded carotenoid signals of different bacterial species in aqueous suspensions with only minimal or even no fluorescence interference. Even chromobacteria species with very similar carotenoid compositions could be distinguished because of the position of the C=C stretching vibration [74]. Additional investigations showed that even a suspension diluted to 1–3 cells generated RR spectra with a satisfactory signal-to-noise ratio [75]. Drawn on this experience, also other working groups studied carotenoid-producing bacteria by means of Raman spectroscopy: *Mycoplasma pneumoniae* strains could be subdivided into those that produced the chromophore and those that did not [76]. In the same context, it was shown that the carotenoid signals of *Staphylococcus aureus* and *M. pneumoniae* are very similar, while the carotenoid level within the species can be highly variable [76, 77]. Also, the enhanced signals of the polyene chain of carotenoids allowed a separation of Gram-positive and Gram-negative plant-associated bacteria such as *Paenibacillus validus* and *Xanthomonas axonopodis* [78].

Even halite and sulfate evaporate rocks have been analyzed by means of RR spectroscopy in search of carotenoid signals from halophilic archaea. The detection of biomolecules that are known components of microorganisms adapted to these extreme settings (high salinity and basic environment) could be good markers for life detection on Mars [73]. In this context, Marshall *et al*. [73] collected the RR spectra of bacterioruberin, which is the most important carotenoid in halophilic bacteria next to β-carotene, directly from *Halobacterium salinarum* samples. A similar study performing Raman spectroscopy with 514-nm laser excitation to find biomarkers in halophilic bacteria demonstrated that carotenoid signals can be detected in lyophilized bacteria [79]. Additionally, the use of 785 nm as excitation wavelength allowed assessing the presence of different osmotic solutes, for example, sugar or amino acids, within stratified microbial communities that occur, for example, in hypersaline systems of sediments [79, 80].

Aside from the analysis of carotenoid signals, the knowledge about bacteria producing cytochrome allowed their discrimination on the species level via Raman spectroscopy: Already in 1985, the RR spectra of different bacterial cytochromes were compared to each other and species-dependent spectral variations were recognized [81]. Also, RR studies on *Mycobacterium tuberculosis* were performed to

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gain a better insight into the expression of the CYP121 gene, which encodes a cytochrome P450 enzyme [82].

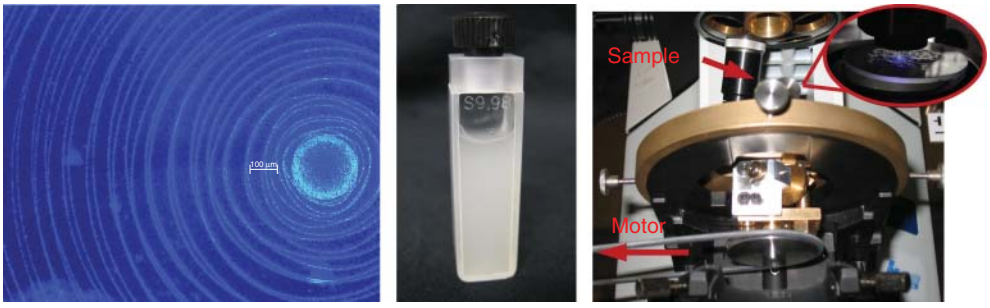
The use of visible wavelengths to enhance the Raman signal of chromophore-producing microorganisms offers the opportunity to analyze or identify these species in a fast and easy way [74, 83–85]. For example, the heterogeneity of microcolonies of the pigmented bacterium *H. salinarum* was analyzed [54], as well as the effects of fixation of purple non-sulfur bacteria such as *Rhodobacter* spp. or *Rhodopseudomonas* [86]. Additionally, the discrimination potential of HCA performed on the RR spectra of these bacteria was investigated. It was shown that discrimination between spectra from different chromophores can be performed easily and reliably, but correct clustering of single-cell spectra containing similar chromophores was obtained only using the weighted average linkage method [87].

RR techniques can also be applied to gain deeper insight into morphology and growth processes. Walter *et al*. [88] exploited the electronic absorption of cytochromes at 532 nm to measure its distribution inside hyphae of *Schizophyllum commune*. From the RR scans of the hyphae and their comparison with other imaging techniques, it was deduced that hyphal tips and branching points contain mitochondria with particularly high amounts of cytochrome c. This confirmed these positions as active growth zones of the fungus.

Therefore, RR spectroscopy performed with visible wavelengths is a useful tool to discriminate and identify microorganisms by analyzing and studying their intrinsic chromophores for large colonies as well as for low amounts of cells. However, important limitations to this classification strategy have also been given. Bleaching effects of the chromophores upon prolonged exposure to the excitation laser light lead to a reduced signal intensity of the resonance enhanced components. This prevents quantitative analyses. In addition, the occurrence of the same pigments in various species restricts discrimination between these kinds of bacteria.

6.4 UV-Resonance Raman Spectroscopy

Shifting the excitation laser into the UV region significantly changes Raman spectra of microorganisms in different ways. Firstly, the signals of macromolecules, whose electronic absorption bands coincide with the wavelength of the ultraviolet excitation laser, are selectively enhanced by a factor of up to 10^6. In biological samples, this applies mainly to aromatic compounds such as DNA and RNA bases or aromatic amino acids. This results in simplified and more specific spectra with fewer signals (see also Figure 6.1 spectrum e). Another advantage of UVRR is the absence of fluorescence in the spectra. It is reported that deep UV excitation has only small occurrences of fluorescence background at 257 nm excitation (due to the aromatic amino acids) and it is negligible for the excitation wavelength being in the region between 190 and 250 nm [89–92]. Since water has only weak vibrational signals at these wavelengths, performing measurements in aqueous



(a) (b) (c)

Figure 6.6 UVRR measurements on microbes. In (a), the fluorescence microscopic image of a burning lane after high-power laser irradiation is shown. Panel (b) shows a typical sample solution in a quartz cuvette. Panel (c) shows a rotation device, which constantly moves the sample on a spiral path during the measurement. The rotation device is set below the sample. The sample shown in (a) was rotated with this device and shows the resulting spiral pathway.

solutions is possible. This point is very important because UV irradiation transfers large amounts of energy to the sample, which can lead to its denaturation and ultimately destruction by burning. This can be seen in Figure 6.6a, which shows a microscopic image of a bacterial film that had been subjected to UV laser irradiation. The bright lines in the image are the lanes where high laser intensity was applied and the sample was burned. To avoid these side effects, UVRR measurements of biological samples are often performed in aqueous environment, usually in cuvettes (Figure 6.6b), as the heat can rapidly dissipate into the surrounding solvent. It should be noted that special fused quartz cuvettes have to be applied, since normal glass absorbs UV irradiation. Also, scattering effects of the bacterial suspension have to be taken into account, which sets an upper limit for cell density in the sample. To avoid both the burning effects on dried samples and the limitations of measurement in suspension, UVRR measurements can be performed on dried films that are constantly moved. Figure 6.6c shows a rotation device that moves the bacterial film on a spiral path during the measurements.

Despite these restrictions on sample measurements, UVRR spectroscopy is highly suitable and interesting for microbial research [90, 93–95].

The first UVRR spectra of bacteria were published by Dalterio *et al*. in 1987 [96]. They measured five different species with an excitation wavelength of 242 nm and proved the main signals to be due to nucleic acids and aromatic amino acids. Different batches from one species gave reproducible spectra, but each of the five investigated species was found to give a distinctly different UVRR spectrum, which could be used for discrimination. Also, they detected spectral variations in *Bacillus* cultures of different age, and ascribed these variations to increasing amounts of CaDPA (calcium dipicolinate) due to spore formation in the cultures.

Further experiments by Britton *et al*., Baek *et al*., and the group of Nelson investigated the dependence of the spectral information on the excitation wavelength for different bacterial samples [89, 91, 97]. They found that shifting the wavelength

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within the ultraviolet region between 222 and 251 nm results in the enhancement of different components of the microorganisms. Applying lower wavelengths, specifically 231 and 222 nm, results in UVRR spectra dominated by aromatic amino acids. Excitation at higher wavelengths of 242–251 nm leads primarily to the enhancement of nucleic acid modes. Chadha *et al*. [98] demonstrated this wavelength selectivity very impressively using purified ribosomes. Using a 222-nm excitation wavelength, the ribosomal spectra displayed nearly exclusively signals of proteins, while excitation with 242 nm gave spectra dominated by nucleic acid bands.

Of course, this selectivity also applies to other compounds. For example, Baek *et al*. measured the UVRR spectra of cyanobacteria using four different excitation wavelengths (222, 231, 242, and 251 nm). With an excitation wavelength of 251 nm, they observed an intense signal which they attributed to quinone, whose signal was not present in the measurements with the other excitation wavelengths [97].

This spectral selectivity of UVRR is so strong that Wu *et al*. [99] were able to show that bacterial spectra obtained with an excitation wavelength of 251 nm are mostly a sum of signals from pyrimidine and purine bases. Based on the knowledge of the amount of all five nucleic bases and their respective Raman cross-section, they calculated the UVRR spectra of *E. coli* and found them to be in good agreement with real spectra. Furthermore, they observed that, in the spectra obtained at longer excitation wavelengths (244–251 nm), the band area of the signal at 1480 cm^{-1} was proportional to the total adenine and guanine content in the cell [100]. Similarly, Nelson *et al*. calculated the UVRR spectra of *Bacillus* spores. They found the real spectra to differ from the calculated ones mostly due to CaDPA signals. So they determined the Raman cross-section of CaDPA as well, and estimated the CaDPA content of the spores from the spectra [101]. In a different study, Baek *et al*. [102] showed that, by selectively enhancing protein-related signals using an excitation wavelength of 231 nm, UVRR is sensitive enough to detect alterations due to protein denaturation for lysozyme and *Staphylococcus epidermidis*. These investigations indicate that the wavelength-based selectivity of UVRR can be exploited to semiquantitatively determine or monitor specific components in bacteria.

Apart from selectively observing certain cell components, UVRR also can be used to study the differences between bacteria. The group of Nelson also investigated several Gram-positive and Gram-negative bacteria as well as endospores of *Bacillus* species with different excitation wavelengths ranging from 222 to 251 nm [91]. They were able to distinguish vegetative *Bacillus* cells from endospores based on the signals of CaDPA. They also observed more subtle differences between spectra of Gram-positive and Gram-negative bacteria. In follow-up studies, they investigated both effects more closely. Using an excitation wavelength of 242 nm, they were able to distinguish between dormant and germinating spores as well as vegetative cells, mainly due to changes in the CaDPA signals [103]. The investigation of *E. coli* and *S. epidermidis* using deeper UV wavelengths of 200 and 218 nm revealed that the signal at 1556 cm^{-1} is strongly related to the Gram type, with the

Gram-negative *E. coli* showing a much more intense signal than the Gram positive *S. epidermidis* [98].

To take advantage of computer-supported analysis in order to fully exploit the subtle differences between UVRR spectra of different bacteria, Simpson and colleagues introduced spectral preprocessing of UV Raman data [104]. They discussed preprocessing approaches in order to minimize day-to-day variations between the spectra, remove shifts from the data, and subtract the water band from spectra. They demonstrated the effectiveness of their approach by applying it to a dataset of protein spectra and determined the amount of different structural motifs such as α-helices and ß-sheets from the UVRR spectra. Investigations like these on how to preprocess UVRR spectra in order to standardize the data paved the way for applying statistical analyses on UVRR data.

By applying spectral preprocessing to their UVRR spectra, Lopez-Diez and Goodacre [90] successfully used multivariate statistical methods to classify six different species of the *Bacillus* and *Brevibacillus* family by their UVRR spectra. Furthermore, by performing an HCA on their UVRR data, they found the resulting dendrogram to be in good agreement with the 16S rDNA classification tree. Jarvis and Goodacre [105] used an excitation wavelength of 244 nm in combination with clustering algorithms to discriminate different bacterial species causing urinary tract infections. Tarcea *et al*. [92] classified nine different species relevant in clean room environments from their UVRR spectra using a support vector machine (SVM). Gaus *et al*. [106] tested several supervised and unsupervised statistical methods and successfully classified three species of lactic acid bacteria on the strain level with either method.

UVRR does not only have the potential to distinguish between species; also the effect of environmental parameters can be monitored. Manoharan *et al*. [107] investigated changes in the UVRR spectra due to various growth conditions such as medium or growth rate.

Furthermore, also the molecular interaction of antibiotics with bacteria, which is of interest, for example, in pharmaceutical research, has been studied with UVRR. For example, Neugebauer *et al*. treated samples of *Bacillus pumilus* and *S. epidermidis* with different concentrations of the fluoroquinolone drug ciprofloxacin and the gyrase-inhibitor moxifloxacin. They observed spectral changes upon exposure to both antibiotics and were able to clearly differentiate between treated and untreated organisms by applying multivariate data analysis [108, 109]. Using an excitation wavelength of 244 nm, Walter *et al*. [110] distinguished antibiotic-treated from nontreated *E. coli* DH5α using SVM and LDA (linear discriminant analysis) models. The results were successfully validated by predicting an independent dataset. They were also able to discriminate plasmid-bearing *E. coli* from plasmid-free ones. As drug resistances often are coded on plasmids, the capability to identify plasmid-bearing bacteria poses a step toward the application of Raman spectroscopy for the determination of bacterial resistances.

Since identification of bacteria is of major interest, but their cultivation and potentially necessary separation steps from accompanying matrices are time

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consuming, the possibility to investigate bacteria directly in a matrix would be of great interest in many areas. Harhay and Siragusa [111] developed a method that allowed them to investigate bacteria directly in a complex food matrix. Using hydrogen–deuterium exchange in combination with UVRR, they were able to resolve the spectral fingerprint of individual components in their mixture and postulate that the detection and identification of microorganisms in food samples may be possible with this technique within a few minutes. Another approach has been proposed by Grun *et al.* [85]. They recorded 2D UVRR spectra of five bacterial species by serial measurements of the same sample while tuning the excitation wavelength between 210 and 280 nm. A modified optical real-time adaptive spectral identification system then predicted the abundance of each of the five species from these 2D spectra with satisfactory accuracy. They postulate this method to be transferrable to real-world samples to detect and identify bacterial content in complex matrices.

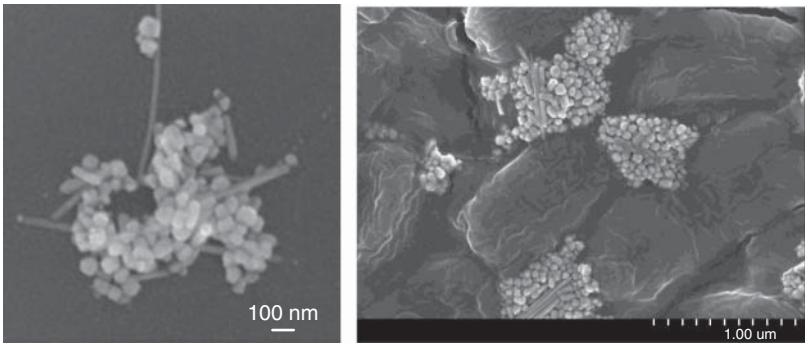
Current UVRR depends on sufficient bacterial biomass for destruction-free analysis. It is worth noting, though, that Chadha *et al.* [112] successfully applied UV excitation within a micro-Raman setup to measure small numbers of bacteria. By cooling their sample to avoid degradation, they were able to acquire spectra of reasonable quality from as few as 20 cells within 15 s.

6.4.1 Surface-Enhanced Raman Spectroscopy

6.4.1.1 The SERS Effect

Apart from the resonance effect, the intrinsically low sensitivity of Raman spectroscopy can be increased by exploiting the enhancement effect of plasmons from metallic NPs, which is named surface-enhanced Raman spectroscopy (SERS). Molecules in very close proximity (i.e., <10 nm) to the NPs experience a strongly increased electromagnetic field as a result of the excitation of plasmonic resonances of the NP, which leads to an enhancement of the Raman signal by factors of 10^3–10^6 [113] and maybe as high as 10^{14} in certain experiments [114, 115]. This increase in Raman signal intensity leads to an increased sensitivity, allowing the measurement of very low concentrations or, in the case of bacteria, fast measurement with high signals. A further advantage of using SERS on biological samples can be gained by the fluorescence quenching effect of the metallic NPs, which in turn reduces the fluorescence background often encountered in biological samples.

However, one major concern in the application of SERS on a routine basis is the reproducibility of the SERS substrates. The enhancement factor and enhancement characteristics strongly depend not only on the size and shape of the NPs but also on their state of aggregation and the presence or formation of so-called "hot spots" [113, 114]. Since they are easy to produce, colloidal NPs are applied most often, even though their enhancement characteristics



(a) (b)

Figure 6.7 SEM images of typical silver colloids. Spherical NPs, rods, and long needles varying in size and diameter are visible. Panel (a) is reproduced from Ref. [114], Copyright (2012) Springer. Panel (b) is reproduced with permission from Ref. [113], Copyright (2004) American Chemical Society.

are least controllable. They can be easily produced in large quantities by the chemical reduction of (cheap) precursors in solution. Also, sample preparation is fairly easy. The colloid is mixed with a bacterial suspension, and measurements can be performed directly on the suspension, or it can be spotted onto suitable substrates and measured after drying [116]. However, regarding the size and shape of the NPs, these reactions can be controlled only to a very limited degree. In Figure 6.7, the scanning electron microscopy (SEM) images of typical silver colloids are shown. Variations in the shape and size of the individual NPs are obvious, which will lead to variations in the enhancement characteristics.

This also leads to variations in the particle mixture between different batches of colloids even if they are produced under the same conditions. Some publications report the UV–vis absorption spectroscopy and electron microscopy characterization for multiple batches of one colloid showing differences in particle size, aggregation state, and particle size distribution [113, 117]. These different batches of colloids will have different enhancement characteristics, and spectra of the same sample obtained using these different batches of NP will differ from each other. Also, the aggregation of the colloidal NPs to form the necessary hot spots can be controlled only to a limited extent, but has an effect on the enhancement and therefore on the spectra. The combination of all these factors introduces large variety into SERS spectra obtained with colloidal NPs, which has prevented their routine application for bacterial analysis so far. Therefore, a major step toward the successful application of SERS to routine bacterial measurements is the development and manufacturing of reproducible SERS substrates. Numerous efforts regarding this task have been made over the last years, but they will not be discussed here. For more information, the reader is referred to review articles on the subject [114, 118–120].

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6.4.1.2 Analysis of Bulk Bacteria Samples with SERS

Despite the challenging issue of spectral reproducibility when using NPs, there have been numerous investigations on bacteria by applying SERS. Two main approaches can be observed: One utilizing SERS labels merely as a sensitive readout method; the other is the direct measurement of SERS spectra from bacteria.

In the first case, usually a Raman reporter dye is coupled to a SERS NP. The reporter itself presents strong and distinct Raman features, of which only a few need to be taken into account, and is therefore easy to detect even in low quantities. This labeled SERS substrate can bind to the target bacteria (either specifically via antibody grafting, or unspecifically), and the bound particle is monitored with Raman spectroscopy. Since not the sample itself will be measured but only the Raman reporter dye, whose signals are strongly enhanced by the SERS particle it is bound to, these spectra do not contain any information of the bacteria themselves. However, with antibody-targeted binding of the NPs to specific bacteria, this setup can be suitable for screening applications since it enables very low detection limits. For example, Wang *et al*. [121] successfully determined bacterial loads as low as 1000 cfu/ml of *S. enterica* and *S. aureus* from different food matrices. Yakes *et al*. used bacterial lysate of *Mycobacterium avium* subsp. *paratuberculosis* to determine the amount of bacterial-specific proteins with antibody-mediated capturing of the species-specific proteins. They obtained detection limits down to 500 bacilli per milliliter [122]. Antibody-targeted addressing of SERS NP has also been applied to investigate virus samples. For example, the group of Driskell *et al*. [123] successfully detected feline calicivirus within culture media using Raman-labeled gold NPs.

Apart from antibody-targeted methods, quantification is also possible using colloidal SERS substrates on the bacteria themselves. By scanning an area of their sample, Knauer *et al*. [124] were able to determine the bacterial load in a working range of $10^6 - 10^9$ cfu/ml for *Legionella pneumophila* and $10^8 - 10^9$ cfu/ml for *Salmonella typhimurium*. Kastanos *et al*. [115, 125] also established a method to determine the bacterial concentration in the range of $10^3 - 10^8$ cfu/ml. They even could distinguish between *E. coli* samples with a bacterial load lower than 10^4 cfu/ml and those with a load higher than 10^5 cfu/ml with an accuracy of ~82%. Such threshold distinctions are of importance in the detection of infections such as urinary tract infections, where bacterial loads higher than 10^5 cfu/ml are considered to be an infection. Wang and colleagues successfully measured SERS spectra from bacterial concentrations as low as $10^2 - 10^6$ cfu/ml [126].

Owing to their easy fabrication, colloidal NPs have been applied most often for SERS investigations of bacteria. Aside from the problem of reproducible sizes and shapes, the aggregation state is of great importance for the achievable enhancement. It can be triggered by the addition of different salts. For SERS measurements on bacteria, also the relative position of the aggregates to the bacteria is important. Knauer *et al*. [124] showed in their work that the obtainable SERS signal of bacteria strongly depends on the deposition method of the NPs. Application of agglomerated NPs results in only a very limited efficiency to detect bacteria, whereas if the

agglomeration of the colloidal SERS particles took place directly on the bacteria, more than 90% of the visible bacteria gave suitable SERS spectra. This is in accordance with the reports of Zeiri and Efrima, who developed a new method to reproducibly produce and deposit NPs at bacterial cells [127]. In a two-step approach, they first impregnated the bacteria with one of the two solutions needed for colloid formation: Either the metal salt or the reducing agent. After washing the bacteria and applying the second solution, the NPs were formed exclusively at the bacteria. With the order of reagent application, a directed deposition of the NPs onto the surface or into the bacterial cells was possible. This approach minimized spectral distribution from the surrounding matrix and simultaneously yielded very reproducible spectra. Additionally, it allowed the determination of which information one will get from the spectra by the choice of the NP localization.

By applying different SERS substrates, numerous groups have shown that SERS spectra of different bacterial species show different spectral features, which allows the qualitative differentiation of the species by their SERS spectra [113, 117, 120, 124, 126, 128–140]. The range of the investigated bacteria comprises Gram-positive as well as Gram-negative bacteria. Often, both are represented in the same study [113, 120, 126, 130–136, 139], but not necessarily so. It is worth noting, though, that sometimes different spectra are reported for the same species [120, 127]. This is mostly due to the fact that SERS is very sensitive to the spectral acquisition parameters. Particularly, the localization of the NPs at the bacteria seems to be crucial. This is not surprising because SERS is known to be effective over a few nanometers only. For preaggregated colloids, the hot spots most likely will be within the aggregates and not in the necessary closeness to the bacterial surface. NP aggregation directly in the presence of bacteria will presumably lead to more hot spots on the surface and therefore a stronger signal. Still, drying of NP–bacteria suspensions on substrate slides is known to give inhomogeneous films and therefore fluctuations in the spectra [127, 141]. For aggregation or formation of NPs directly in the presence of bacteria, the affinity of some metals to certain chemical structures may have to be taken into consideration. Efrima *et al*. discussed in great detail the affinity of silver to FAD (flavin adenine dinucleotide) and the consequences for their bacterial SERS spectra [142]. Finally, because of the enhancement being restricted to a very close vicinity, SERS particles localized within a bacterium will give distinctly different spectra from those on the outside [127, 142].

In the end, mostly because of the difficulties of obtaining reproducible SERS spectra from colloidal NPs, only a few groups have attempted to distinguish between different species, and often only classifications have been carried out. Different approaches have been applied for this task. Liu discriminated between two species by visual inspection, comparing two peak intensity ratios [133]. Wang *et al*. [126] distinguished three pathogenic species by classifying them with canonical variate (CV) analysis. The group of Zhang applied principal component analysis (PCA) for the classification of different bacteria [138]. Patel *et al*. [135] used PCA as well; not only did they succeed in the differentiation of bacteria by classification, they were even able to correctly assign the left-out samples with

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this approach. Chu and colleagues applied PCA to classify different bacterial species. They could even discriminate three strains of *E. coli* from each other by simple PCA classification [130]. Some groups have investigated mixed bacterial samples and were able to tell them apart from pure cultures by PCA [130, 143] or Euclidian distance [128, 129]. Surprisingly, the mixed samples did not appear in the area between the pure samples but were shifted toward one constituent of the mixture [129, 143] or even showed larger difference from both original components [128, 130].

Only a few more sophisticated statistical analyses on SERS spectra have been carried out. Interestingly, they all deal with distinguishing very closely related groups, namely different strains of one species. For example, Jarvis *et al*. [131] also applied PCA, but they combined it with discriminant factor analysis (DFA) to differentiate nine closely related *Bacillus* strains or five strains of *E. coli* [113]. In contrast to most other groups, they did not only classify the spectroscopic data but also validated their models with left-out replicates [131] or completely independent batches of their samples [113]. Hennigan and colleagues distinguished three strains of *M. pneumoniae* with PCA, HCA, and PLS-DA classification [144]. Furthermore, they discriminated between infected and noninfected samples by applying CV analysis. The applicability of the CV model was validated with throat swabs spiked with *M. pneumoniae*. Even real clinical swabs were correctly identified as being healthy or *Mycoplasma* infected. Stephen *et al*. [145] applied PCA in combination with LDA to classify 14 different *Arthrobacter* strains. Walter *et al*. [141] utilized an SVM to successfully classify spectra of nine different strains of *E. coli* using internal cross-validation.

It should be noted that only very few of these groups report the impact of different batches of the applied SERS substrate on their SERS spectra [113, 130, 143, 144], and most used either novel SERS substrates [130, 144] or formed the colloidal NPs directly inside the cells [143].

One method to increase SERS reproducibility also for colloidal NPs is using microfluidic devices in a lab-on-a-chip (LOC) setup (Figure 6.8). These devices offer a well-defined detection environment and the LOC provides more constant aggregation behavior as well as homogeneous colloid distribution, both traits that favorably influence the spectral reproducibility. Using such a microfluidic device, Walter *et al*. [141] obtained SERS spectra of *E. coli* that were sufficiently reproducible to perform sophisticated chemometric analyses on the data (spectrum iii in Figure 6.1). Similarly, Lu *et al*. [146] were able to discriminate between methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) based on LOC-derived SERS spectra. They correctly identified *S. aureus* strains from hospital outbreaks to be MRSA or MSSA. Furthermore, also quantification of MRSA was possible from an MRSA/MSSA mixture. It is worth noting that the experiments of Li were carried out at two different locations with different instruments using different batches of the colloid. Therefore, LOC-SERS can alleviate the disadvantages of colloidal SERS substrates, which would favor its more widespread application for bacterial identification processes.

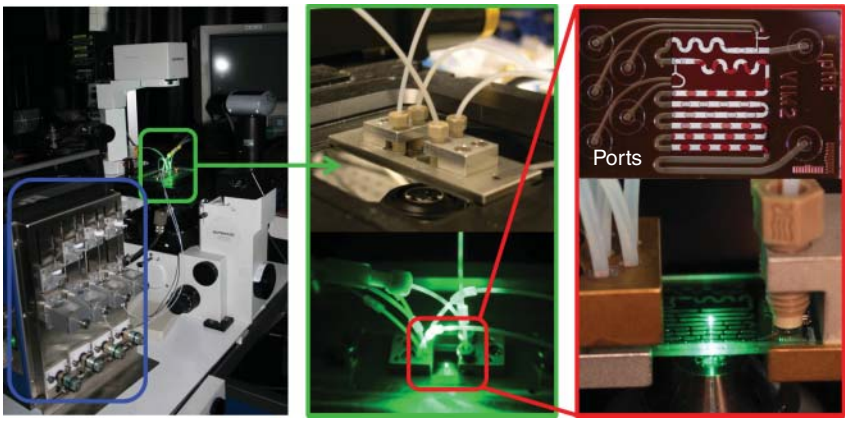

Figure 6.8 Microfluidic setup. The assembled microfluidic setup consists of the syringe pump system (blue), the microfluidic chip (green) mounted onto a microscope, and a laser (not in the picture). The chip is illuminated with a 532 nm laser. On the right side, a close-up of the chip design and the mounting site is shown (red). For segmented flow operation, a mineral oil is pumped through the chip, into which the analyte and other components can be injected via separate ports.

SERS of bacteria is applicable for answering other questions aside from species identification as well. Wilson *et al*. [147], for example, used SERS to detect the pigment scytonemin in cyanobacteria at different concentrations. Stephen *et al*. [145] investigated the influence of culture conditions (solid versus liquid) and increasing chromate concentrations on the SERS spectra of an *Arthrobacter* strain and found significant differences. Liu *et al*. investigated the impact of different antibiotics on bacterial SERS spectra. They could show an influence of the antibiotic treatment on the SERS spectra. Furthermore, the spectral changes depended on the antibiotics' site of action. For antibiotics acting on the bacterial cell wall, these changes were visible as early as 20 min after initialization of the treatment [134]. Kastanos *et al*. [125] also found spectral differences in samples depending on whether they were susceptible or resistant to an applied antibiotic. These findings suggest that SERS could provide the possibility of fast detection of antibiotic susceptibility.

Also, virus detection benefits from reproducible SERS substrates. Alexander used commercial reproducible SERS substrates to distinguish between bovine papular stomatitis viruses and pseudocowpox viruses using PLS [148]. He successfully validated his model with independent viral samples. Driskell and colleagues were able to detect rotaviruses in a complex matrix of cell debris and virus particles using areas of oblique-angle-deposited silver nanorods as NPs [149]. Using the same substrate, Negri *et al*. [150] detected influenza viruses by capturing them with specific oligonucleotides that were bound to a SERS-active surface and by monitoring the spectral changes that occurred upon binding. In both reports, the spectral reproducibility was high enough to perform statistical

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analysis on the data and discriminate between different viral strains. Driskell *et al*. [149] were even able to quantify the viral load of the samples.

In summary, SERS on bacteria is a promising approach but is not suitable for standard application yet. Currently, it suffers from reproducibility issues due to the variations between NP batches. To solve this problem, colloidal NPs may be used in microfluidic devices. Also, efforts to generate SERS-active substrates reproducibly have been made by several groups, which resulted in solid substrates that showed good spatial homogeneity in the enhancement characteristics [114, 118–120]. With such substrates, the spectral reproducibility that is necessary for bacterial (or viral) distinction can be achieved, as has been shown by several groups already. Given that external SERS substrates will yield information on the exterior of bacteria and that numerous pathogenicity markers (e.g., for serum classes) are located on the bacterial surfaces, SERS may provide a method to quickly do strain typing of pathogens. Therefore, the advancement on SERS substrates will permit increase in research activities using SERS on bacteria and more applications will surely follow.

6.5 Single-Cell Analyses

Because for the investigation of bulk material the samples are usually precultivated, standardization of the samples by applying the same cultivation conditions is easy. This results in a good reproducibility of the IR and Raman spectra. On the other hand, cultivation takes at least 6 h to grow microcolonies with $10^5 - 10^6$ cells. For some bulk measurements, even higher cells numbers and hence longer cultivation times are required.

Micro-Raman setups with visible excitation wavelengths can achieve a spatial resolution in the size range of single bacteria. Therefore, the analysis of single cells is possible [151]. However, the investigation of single cells poses some questions that need not to be discussed for bulk material, but will be highlighted in the following section.

6.5.1 Micro-Raman Spectroscopy with Excitation in the Visible or NIR Region

The single bacterial cells have to be brought on a Raman-compatible substrate such as fused silica, calcium fluoride, or nickel to measure the Raman spectra. Depending on the aim of the investigation, they can be extracted from their original matrices or measured directly inside the matrices such as, for example, biofilms.

The first analyses of single bacterial cells were performed by Schuster *et al*. [152, 153]. They evaluated the Raman spectra of *Clostridium* cells from an acetone–butanol fermentation process with respect to bacterial composition. Using a Raman excitation wavelength of 633 nm, they carried out a line scan over

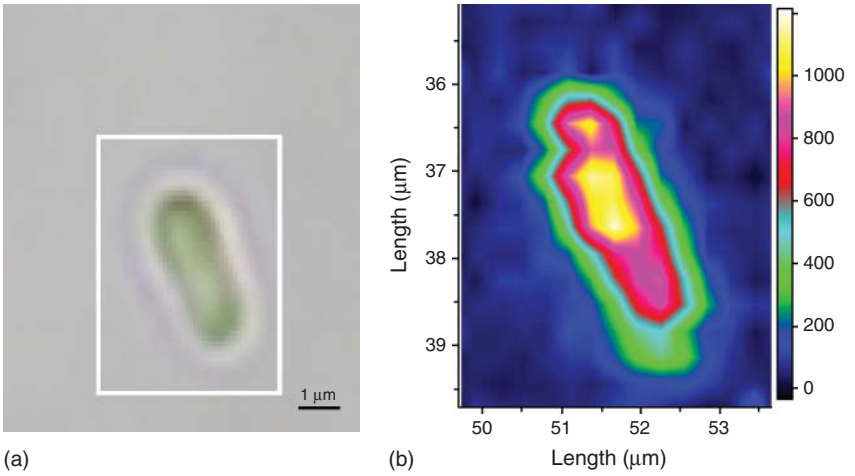

Figure 6.9 Raman mapping over a single *B. sphaericus* cell. (a) Microscopic image and (b) Raman false color plot of the CH stretching vibration. Figure is adapted from Ref. [154], Copyright (2005), American Society for Microbiology.

the long axis of the cell showing the difference between background material and bacteria and – more importantly – no chemical changes inside the cell [152].

Further Raman mapping experiments on single bacterial cells by other groups proved the spectroscopic homogeneity of bacteria. In Figure 6.9, a microscopic image of a *Bacillus sphaericus* cell (Figure 6.9a) and its corresponding Raman map (Figure 6.9b) are displayed [154]. In the map, the signal intensity of the CH stretching vibration is visualized. As shown by Schuster, in one dimension [152] the bacteria can be clearly distinguished from the background; they also show a homogeneous distribution of the CH stretching vibration but also other signals throughout the entire cell. Therefore, a single Raman spectrum is representative for a single bacterial cell.

This spectrally homogeneous composition of microorganisms was also observed by Esposito *et al*. [155] upon mapping bacterial spores. Using 488 nm excitation wavelength and photobleaching the spores for approximately 1 min prior to Raman measurement, the Raman spectra of endospores from four different *Bacillus* species were analyzed, showing identical signals of CaDPA and proteins as well as a homogeneous distribution of the components within the spores.

Although the investigation of single bacterial cells may result in spectra with lower signal-to-noise ratio because of the reduced sample volume, it offers numerous advantages. For example, Raman spectra of single bacterial cells often show a reduced fluorescence background when compared to bulk spectra because, during bulk measurements, contaminations from various sources (such as the cultivation medium) may interfere with the measurement [154].

Also, the measurement of individual cells allows access to the heterogeneity in a genetically homogenous microbial community *in situ* or investigation of individual particles in a complex matrix. For example, Schuster *et al*. [153] observed

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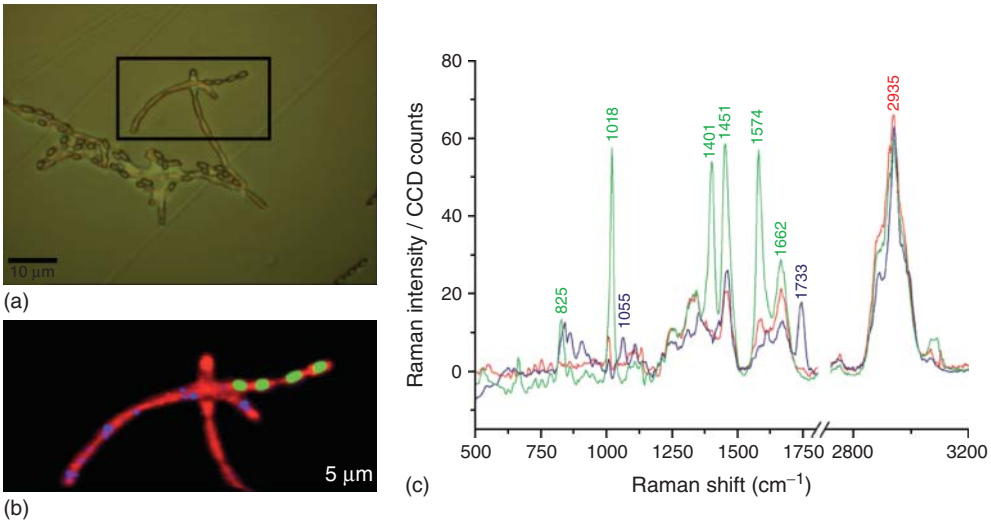

Figure 6.10 (a) Bright-field microscopic image of a *B. cereus* cell population. (b) Raman false color image showing the integrated Raman intensity of the ν (C=O) stretching vibration at 1726 cm^{-1} (blue – PHB), of the CH-stretching intensities in the 2800–3000 cm^{-1} region (red – cell), and the symmetric ring "breathing" of pyridine at 1018 cm^{-1} (green – spore) of the area shown in (a). (c) Corresponding Raman average spectra of the chemical image (b). The figure is reproduced from Ref. [156] with permission of The Royal Society of Chemistry.

varying amounts of storage material (granulose) inside different cells of the same culture. Hermelink *et al*. [156] imaged the inhomogeneous distribution of the storage materials poly-β-hydroxybutyric acid (PHB) and CaDPA of endospores in vegetative cells of one sample of *Bacillus cereus* (Figure 6.10).

To analyze particles of interest in a complex matrix, Pätzold *et al*. [157] applied micro-Raman mapping with 532 nm excitation to localize the anammox bacteria *Brocadia anammoxidans* between calcium phosphate and algae inside a biofilm. Also, they successfully localized colonies of anammox bacteria and *Nitrosomonas* inside biofilm granules due to the presence of cytochrome *c* [70].

Apart from these variations within one sample, of course also the growth parameters influence the Raman spectra of single bacteria just like they influence the spectra of bulk samples (Section 6.2.2). In their work, Schuster *et al*. [153] not only detected varying amounts of storage material (granulose) inside the cells but also found spectral variations for different batch ages. More specifically, they observed a decrease in the intensity of the amide I band with respect to the lipid signal. Huang *et al*. detected spectral variations due to the growth phase the bacteria were in [158].

This influence of growth parameters on the Raman spectra may even be used to quantify the said parameters. For example, Walter *et al*. [159] grew a *Streptomyces* species under different nickel concentrations and found dose-dependent relations in the spectra of single cells. Moritz *et al*. [160] monitored the spectroscopic

change upon antibiotic treatment of bacteria and found time-dependent alterations. Huang *et al*. [161] also observed a significant shift of protein and nucleic acid bands after a ^{13}C uptake by bacterial cells. For example, the band ratios of the phenylalanine peak at 1003 cm^{-1} (unlabeled) to the ^{13}C-labeled one at 967 cm^{-1} correlate to the ^{13}C content in the growth medium. This correlation could be used to determine the ^{13}C content in environmental samples. These findings suggest that the measurement of Raman spectra of single cells offers the possibility to determine or monitor environmental parameters such as heavy metal concentration, isotope ratios, or others.

Another area of interest is the investigation of biofilms and their formation (see also Section 6.2.1). Pätzold *et al*. [157] applied micro-Raman mapping to localize bacteria in an intact biofilm. Huang *et al*. were able to distinguish planktonic bacteria from those recovered from *Pseudomonas fluorescens* biofilms. They observed changes due to different oxygen concentrations in the medium [162]. Andrews *et al*. [163] also compared planktonic and biofilm-derived cells. They isolated planktonic cells and biofilms of *Rhodococcus* and *Sphingomonas* from the soil subsurface and from a contaminated groundwater aquifer. Carotenoids, which protect cells against oxidative damage, were identified in planktonic cells but not in cells derived from biofilms. Using Raman microspectroscopy, it was also possible to determine which compounds play an important role for the attachment of the bacteria to a surface and thus for biofilm formation. For *Rhodococcus*, lipids were found to be a key component for successful surface attachment, while for *Pseudomonas* proteins were found to be vital.

One main interest throughout microbiology, however, is to discriminate between different bacteria. In Section 6.2, we already discussed in detail how bulk bacterial samples can be identified by IR and Raman spectroscopic methods. It would be of great interest to expand this technique to single bacterial cells as well. This would enable the investigation of demanding samples containing only few bacteria of interest or species that are difficult to cultivate or are even non-cultivable at all. Incited by these questions, numerous Raman microspectroscopy experiments were performed to distinguish single cells, mostly with an excitation wavelength of 532 nm, as is reflected by recent reviews on the subject [94, 115, 164].

In Figure 6.11, examples of single-cell Raman spectra of six different species are shown. Similar to the spectra of bulk samples, the differences between these spectra are minor. Therefore, proper evaluation of the spectra requires the application of chemometric methods, again. However, the previously discussed advantages in analyzing single cells simultaneously render the identification of such cells more demanding than the identification of bulk bacterial samples. Since no standardization process takes place for single-cell measurements, the variations within one collective of bacteria as well as between different collectives have to be taken into account during the analysis. In a first step, a reference database has to be established. Later samples will be identified by comparing their spectra with the data in the reference database. For this reference database, numerous single cells have to be measured to cover the biological variability of a sample due to different

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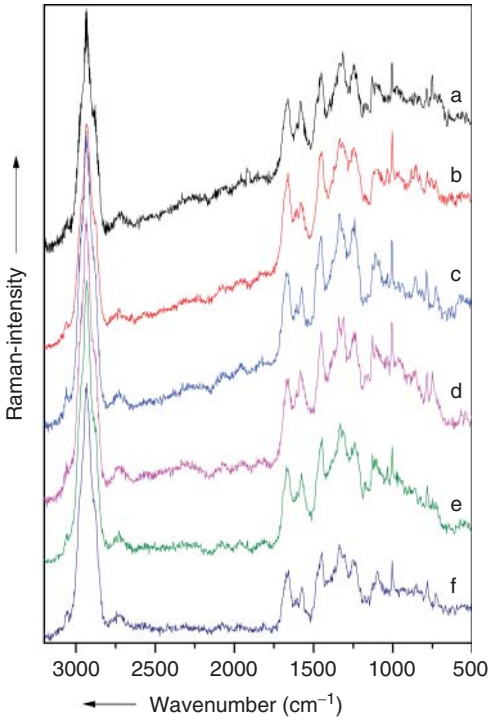

Figure 6.11 Raman spectra of single bacterial cells from the species *Pseudomonas aeruginosa* (a), *E. coli* (b), *Streptococcus pneumoniae* (c), *Staphylococcus aureus* (d), *Staphylococcus saprophyticus* (e), and *Enterococcus faecalis* (f) without spectral preprocessing.

age of the single cells or their position in the cell cycle, for example. Harz *et al*. [165] showed the need to include Raman spectra of bacteria grown under different cultivation conditions in a database of single bacteria for accurate classification.

Because of this necessary increase in spectral numbers, the evaluation of the spectra cannot be performed by mere visual inspection anymore, but statistical methods have to be applied to group the data. An early approach was presented by Huang *et al*. in 2004 [158]. They used PCA, DFA, and HCA to discriminate between single cells of seven species. Later Rösch *et al*. [166] applied SVM to distinguish between 28 bacterial strains belonging to ten different species and assigned unknown samples to their respective strain with an accuracy of 96%. The power of these statistical tools was demonstrated very impressively by Stöckel *et al*. [167] in their investigation of *Bacillus* endospores. Although CaDPA is the main component of all *Bacillus* endospores, the authors successfully identified the endospores of *Bacillus anthracis* and four other *Bacillus* species using a SVM. Kusić *et al*. [168] have shown that also the discrimination between pathogenic and apathogenic *Legionella* spp. was possible. In addition, they were able to classify 22 different *Legionella* species and validate their model with independent batches.

Latest research frequently aims to transfer the approach of Raman spectroscopic identification of single cells to real-world applications. These applications can be very diverse, for example, ranging from water analysis to pathogen identification, biothreat detection, and also food safety issues. Already in 2008, Tripathi *et al*. used micro-Raman imaging to classify waterborne pathogens, although on a very basic level. They distinguished Gram-negative and Gram-positive bacteria from each other as well as from toxins [169]. Only 1 year later, Harz *et al*. [170] accomplished the identification of *Neisseria meningitidis* directly in a meningitis patient's cerebrospinal fluid specimen. Recently, Kloß *et al*. [171] succeeded in identifying bacterial pathogens directly from human urine samples without any previous cultivation. Stöckel *et al*. [172] successfully isolated and identified various *Bacillus* spores from powder matrices. This offers the possibility for the rapid detection of *B. anthracis* spores, which have been used as biological warfare agents in the past [173–175]. Meisel *et al*. [176] implemented a Raman database for the identification of single bacterial cells (*Brucella* spp., *E. coli*, *Ochrobactum* spp., *Pseudomonas* spp., and *Yersinia enterolytica*), which were isolated from spiked milk samples. Similarly, six of the most important harmful bacterial species were isolated from spiked meat samples and identified using SVM [177].

For most of these current applications, the isolation of the bacteria from the respective matrix is necessary. Such isolations may be achieved easily, but they also may pose great problems, especially if only few microorganisms are present in the sample. Therefore, localization of microorganisms in a complex matrix is a highly important but difficult task. Current methods solve this task by combining Raman microspectroscopy with other techniques.

For example, density gradient centrifugation [176] and isolation using antibody-modified substrates to catch bacteria [178] have been shown to be suitable and Raman-compatible approaches. Also, the combination of Raman microspectroscopy with microscopic images and fluorescence imaging allows the localization of bacteria and their differentiation from abiotic particles. Exploiting only the auto-fluorescence of particles, Kalasinsky *et al*. [179] were able to localize single vegetative cells and bacterial spores in a matrix. Based on the Raman spectra of the so-found particles, they distinguished vegetative cells from spores as well as cells of different species from each other. However, the application of auto-fluorescence is limited because abiotic particles may show fluorescence characteristics similar to living cells, for example, polymer particles.

A more selective approach, therefore, is to treat the bacteria with a selective fluorescent stain. Particular care is necessary in choosing the appropriate dye. Since even traces of fluorescence will greatly interfere with Raman spectroscopy, the excitation of the dye has to be at a sufficient distance from the applied Raman excitation wavelength. Krause *et al*. [180] have shown that using the proper combination of fluorescence stains and Raman excitation wavelengths allows the measurement of nearly fluorescence-free bacterial Raman spectra.

The combination of Raman microspectroscopy using an excitation wavelength of 532 nm with fluorescence *in situ* hybridization (FISH) was shown by Huang

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et al. [161]. FISH was carried out with three different labels (Fluos, Cy3, and Cy5) and used to localize the bacteria. Subsequently, a 3-min bleaching step preceded the Raman measurement in order to avoid fluorescence interference of the FISH labels. With this combination of Raman, FISH, and stable isotope probing (^{13}C-labeled compounds), the authors studied the naphthalene biodegradation capability of a ground water community. They identified *Pseudomonas* species to be important naphthalene degraders. In a subsequent study, by applying this combination of techniques they were even able to detect and identify an uncultivable species, which turned out to be an *Acidovorax* species and determine its role in naphthalene biodegradation [181]. This work shows the potential of Raman microspectroscopy to help to detect and identify single cells of organisms.

Another easy yet clever approach to select single particles in a complex matrix is laser tweezers Raman spectroscopy (LTRS). For this technique, the force of a laser beam is used to trap a small particle in the laser focus like tweezers. Such a trapped particle can be manipulated further, for example, by moving it around or by measuring its Raman spectrum. The Raman excitation laser can be chosen freely and may even be the one used for the trapping process. To minimize photodamage, LTRS is often carried out with a wavelength of 785 nm. LTRS may be applied to simply catch and measure single bacteria in a liquid environment. For example, Moritz *et al*. [160] used LTRS to investigate antibiotically treated bacteria in an aqueous environment. Besides bacteria, also yeast cells were measured in optical traps [182, 183]. However, the use of LTRS also allows the differentiation between matrix components and bacterial contamination in complex media. Chan *et al*. distinguished bacterial endospores from a mixture with abiotic particles by trapping single particles and measuring their respective Raman spectra. The endospores were identified by their marker substance CaDPA [184]. Xie *et al*. [185] effectively discriminated bacteria and lipid droplets in spoiled milk samples. Huang *et al*. [186] used LTRS to sort single cells of *E. coli*, ^{13}C-labeled *P. fluorescens*, and the yeast *Saccharomyces cerevisiae* according to their Raman spectra. The sorting results were validated by single-cell genome amplification and cell cultivation.

However, one major advantage of LTRS is the opportunity to keep single cells under constant surveillance. This enables the study of dynamic processes on a single-cell scale. For example, Xie and Li [187] studied single cells of *E. coli* with shifted excitation difference techniques to obtain multidimensional Raman spectra from each cell. They even monitored the heat denaturation on single cells [188]. Chen *et al*. [189] explored the lysis of single *E. coli* cells with two different approaches: The lysis from "outside" with egg white lysozyme, and from "within" by temperature-induced temperate bacteriophage λcI857.

But not only the localization of microorganisms is a challenging task. For large or compartmentalized microorganisms, the investigation of single cells by Raman microspectroscopy is further complicated, because the cell is most likely not homogeneous anymore. This results in different spectra at different measurement positions and hence one spectrum may not be representative of the whole

organism anymore. For example, Walter *et al*. investigated filamentously growing (hyphal structured) *Streptomyces* bacteria at the single-cell level [190]. They cultured two *Streptomyces* species for up to 7 days and performed age-dependent measurements. The lipid content of the cells increased during the cultivation time from day 3 onwards until the lipid signals masked the signals of other cell components. The lipid content was found to differ from one position to another within one cell. Still, despite this obvious heterogeneity of the cell composition, the authors found that a single spectrum of each cell could be used to distinguish between the two species with 91% accuracy. However, this does not necessarily hold true for other samples such as yeasts or fungi. For example, Münchberg *et al*. [191] found that both the lipid content and the lipid composition can vary within intact fungal hyphae.

Apart from those on bacterial cells, also many investigations were carried out with yeast cells. Being eukaryotes, yeast cells exhibit compartments, which results in variations in the Raman spectra obtained at different positions from the same cell [192, 193].

By mapping single yeast cells, Rösch *et al*. [192] showed the heterogeneity of such cells. The results of these mapping experiments are depicted in Figure 6.12. The microscopic image (panel a) is compared to the distribution of proteins (as determined from the amide I vibration at 1624–1687 cm^{-1}, panel b) and DNA (as determined from the signals of DNA bases at 1567–1599 cm^{-1}, panel c). While the proteins are found to be homogeneously distributed throughout each individual cell, the signal from DNA is found to be more intense in one region, namely the position of the cell nucleus.

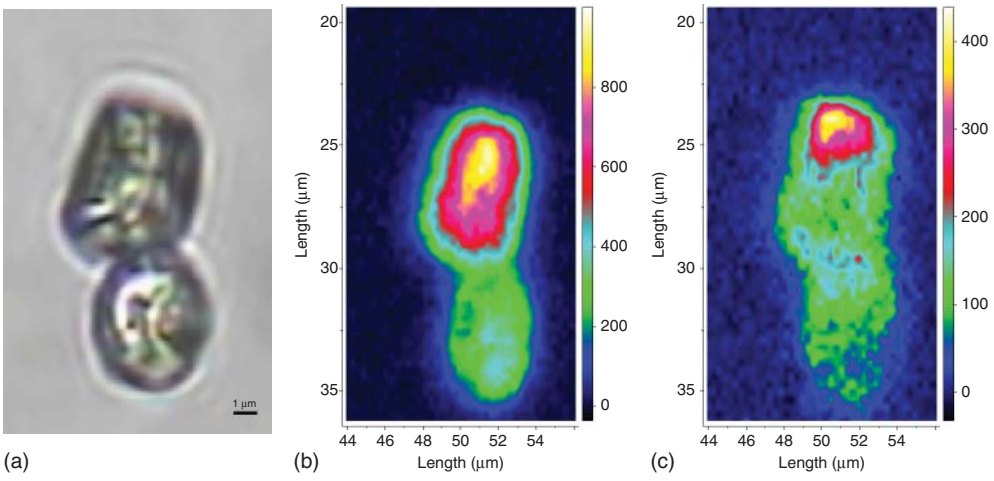

Figure 6.12 Raman mapping over two single yeast cells. (a) Microscopic image; Raman false color plot of the amide I vibration (1624–1687 cm^{-1}) (b) and the DNA signal (1567–1599 cm^{-1}) (c). Images are adapted with permission from Ref. [192], Copyright (2005) John Wiley & Sons, Ltd.

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This spectral heterogeneity requires some attention if yeasts are to be distinguished by their Raman spectra, because one spectrum per yeast cell is not representative of the whole cell anymore. Rösch *et al*. [194] were able to show that by calculating the average from a line scan over the long axis of a yeast cell, one can obtain a Raman spectrum that is representative of the whole cell again, and that can be used to classify single yeast cells. Perhaps not surprisingly, the Raman spectra of yeasts also were found to change corresponding to changes in the growth conditions [195].

Apart from requiring more effort to discriminate single yeast cells from their Raman spectra, the spectral heterogeneity of the cells can also be an advantage, because it offers the possibility to study other effects. Huang *et al*. [193, 196] monitored yeast mitosis by means of Raman microspectroscopy discriminating several cell components. They also followed the poisoning of a living yeast cell with KCN, during which they observed characteristic changes in the Raman signature of the cell [197]. They also found that the application of KCN induces the destruction of the mitochondrial bilayer membrane [193].

In conclusion, single-cell Raman spectroscopy is very useful to investigate the diversity in microbiological systems. It can be combined with various other techniques such as fluorescence staining for imaging of single cells in complex matrices or optical tweezers to manipulate targeted single cells. Raman microspectroscopy in combination with chemometrics can also be used to classify and identify different microbiological species. However, for identification of real-world samples, suitable procedures to isolate the bacteria from the surrounding matrix are required. Also, large databases are necessary to implement as much as possible variations in the corresponding model. Furthermore, for most statistical models, it is obligatory that the species that should be identified is also included in the reference database. Therefore, single-cell Raman spectroscopy is currently not suited to identify entirely new bacterial species. It is, however, suited to distinguish unknown from known bacteria and to identify samples from a given pool of possible species.

6.5.2 Surface-Enhanced Raman Spectroscopy on Single Cells

So far, the application of SERS to single bacterial cells has been limited, partly due to the reproducibility issues of the colloidal SERS substrates.

Another main reason is the challenge in creating SERS-active hot spots specifically at the bacteria. Simple mixing of bacteria with pre-aggregated colloidal NPs leads to a random distribution of the hot spots. Jarvis and Goodacre [113] have shown such mixtures to result in a nonuniform distribution of the colloidal NPs on the bacteria. The occurrence of NP hot spots at the site of single bacteria would not be reproducible and, therefore, also the SERS spectra of different single cells obtained by this method cannot be compared, much less the SERS spectra from different batches. To counter this effect, a large amount of colloidal NP could be applied to the bacteria in order to form a film of NP on the bacteria.

In this way, hot spots on the bacterial cells would be certain, but this method requires large amounts of NPs. Alternatively, the NP and bacteria could be concentrated, as shown by Zhang *et al*. [138]. By applying an external magnetic field to a mixture of bacteria and magnetic NPs spread onto a slide, they were able to mechanically concentrate the bacteria and NPs in a small area. The NPs completely surrounded the bacteria, and a reproducible measurement of SERS spectra was possible.

Specific labeling of the NPs by antibodies could, of course, help to direct the NPs toward single bacteria, and spectra have been obtained by particle-tagged bacteria [121, 139]. But such assays would only be feasible to confirm the presence of those bacteria that interact with the specific NP-attached antibody. All bacteria that do not interact with this antibody would go undetected. Such an approach can be useful, for example, in screening applications for quality control, water surveillance, or food safety, when only the presence or absence of a specific pathogen is of interest.

For the identification of unknown bacteria in samples, the measurement of all bacteria has to be possible. This can be achieved only if NP-generated hot spots can be positioned specifically at the bacteria. One method is to deliver the SERS-active NP directly to each individual cell upon measurement. This is done in tip-enhanced Raman spectroscopy (TERS), where a colloidal NP is attached to an AFM tip. With such TERS tips, AFM and SERS measurements are performed simultaneously, ensuring the presence of a hot spot at the measurement site. Neugebauer *et al*. [198] have investigated single *S. epidermidis* with this approach. Deckert-Gaudig *et al*. [199] characterized the purple membrane of *H. salinarum* and its isolated membrane patches using TERS. It is worth noting that the high spatial resolution provided by the AFM setup also allows the TERS investigation of single virus particles. Corresponding experiments have already been performed by Cialla *et al*. [200] on a tobacco mosaic virus and by Hermann *et al*. [201] on avipox viruses and adeno-associated viruses.

Unfortunately, the setup for TERS measurements is very complex, and the TERS tips are are only available to a very limited extent. Therefore, TERS is not the method of choice to investigate single cells using the SERS effect. Utilizing more robust and more widespread equipment is desirable. To this end, the NPs have to be directed to the bacteria differently. Several approaches have been developed. Dutta *et al*. grew bacteria in the presence of NPs, which was possible because they applied ZnO NPs instead of the frequently used toxic silver NP. This addition of the NPs to the medium led to their incorporation into the bacteria, which in turn enabled SERS investigation of single cells [202]. This approach may be beneficial to investigate single bacteria under laboratory conditions to monitor their development or response to external stimuli. It is, however, not applicable if microorganisms in a given sample are to be detected and identified.

For such a task, the targeted deposition of SERS-active NPs within, on top of, or next to the bacteria in a sample is required. Some groups have made efforts to accomplish this task with different approaches and varied success.

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Knauer *et al*. [124] found that a direction of NP agglomeration to the bacterial surface, and especially to the poles, was possible by the addition of azides. With their method, they were able to successfully measure the SERS spectra of more than 90% of the single bacteria in their sample.

A more stable and feasible solution probably is the directed formation of the NPs on or even within the bacterial cells. Over several years, the group of Zeiri and Efrima has developed a reproducible method to selectively form NPs either on the surface of bacteria resulting in a silver coating, or inside the cells [127]. With this technique or variations thereof, several groups were able to measure the SERS spectra of single bacteria [113, 143].

One early approach toward single-cell detection applying Zeiri and Efrima's technique was presented by Jarvis and Goodacre in 2004 [113]. They produced colloidal silver NPs directly in the presence of bacteria of two different species and prepared monolayered bacterial films from the resulting suspension. Their approach did not lead to SERS-active clusters on all bacteria, and consequently SERS spectra could not be obtained from all microorganisms. However, by combining their micro-Raman setup with SEM, they were able to first determine areas where bacteria and NP were colocalized and measure high-quality SERS spectra from these spots. Because of the monolayer of bacteria on the substrate, they estimated four bacteria to be present in the laser spot per measurement. So the spectra are not exactly single-cell measurements but they come already quite close. The obtained spectra were reproducible enough to distinguish the two species by statistical analysis. Unfortunately, this approach does still depend on the arbitrary occurrence of NPs attached to bacterial cells. Furthermore, this particular approach could only be carried out for single bacterial measurements, if permanent access to a SEM is given, which is rarely the case.

A different route was pursued by Kahraman *et al*. [132]. They secured colloidal NPs to bacteria by coating them with special polymers. This approach prevented the frequently observed aggregation of the bacteria in the presence of NPs [132, 203], and the group was able to characterize single bacteria by SEM (to show the NP distribution on the bacteria) and SERS. The variance of SERS spectra from different bacteria was determined to be just 10%, but only for measurements with one batch of colloid. The batch-to-batch variation was investigated as well, which was much larger. Still, spectral differences between Gram-positive *Staphylococcus cohnii* and Gram-negative *E. coli* were discernible.

In conclusion, SERS investigations on single bacterial cells have been limited to date because of the discussed reproducibility issues of preformed colloidal NPs. The approach introduced by Zeiri and Efrima to generate colloidal NP directly onto or within the bacteria increases the NP reproducibility and also solves the problem of targeting NP to the bacteria. However, this approach requires (repeated) manual handling of each sample to generate the NPs within. Also, the reaction parameters have to be carefully controlled, which may not always be feasible in routine analysis. The efforts in reproducibly manufacturing SERS-active NPs on a surface most likely will bring the measurement of SERS-spectra

from single bacteria a major leap forward. In contrast to NPs generated in the presence of the analyte bacteria, such surfaces are produced independently of the bacterial samples. Samples containing bacteria can then simply be spotted onto these surfaces, and single bacteria may be localized and measured similar to the confocal micro-Raman approach. Because the SERS-active surfaces also provide homogeneous and reproducible enhancement characteristics, reproducible SERS spectra are obtained that are suitable for chemometric data evaluation. Chu and colleagues and Premasiri *et al.* have shown the feasibility to obtain SERS spectra from single bacteria under these circumstances [130, 136]. Liu *et al*. [134] successfully measured single *S. aureus* cells and could even monitor vancomycin-induced changes by sequential SERS measurements. Since extracellular SERS substrates will inevitably provide information on the cell surface, this may offer the opportunity to determine surface-related cellular properties such as serotypes or virulence factors for single bacterial cells. Therefore, single-cell SERS is a promising tool to obtain information from bacteria that are not directly available by current Raman spectroscopic methods, as soon as the main issue of producing reproducible substrates in a large scale is solved.

6.6 Conclusion and Outlook

IR and Raman spectroscopic techniques enable simple, rapid, and accurate classification and identification of bacteria down to the species or even strain level. Measuring times for all techniques are short, and there is no need for expensive chemicals. In addition, large amounts of chemical information can be obtained in a noninvasive and nondestructive manner from only minimal amounts of biomaterial.

For bulk measurements, a large number of bacteria are required, which is normally obtained from a colony or microcolony. In principle, bulk measurements are more stable than single-cell analysis, because they have the advantage of averaging over thousands or even millions of cells. In addition, because of the necessary cultivation procedure, also a standardization of the cultivation conditions can be realized. Therefore, bulk techniques are normally used when the analysis time is not too critical.

Depending on the applied method, the focus of the spectroscopic analysis can be put more on the phenotype (visible and NIR Raman excitation, IR absorption) or on the genotype (UV Raman excitation). Raman spectroscopy has also been proven to be sensitive enough to probe culture conditions and follow metabolic changes during bacterial growth.

With new technical developments, the sensitivity of Raman setups has been increased to detect even single cells, which makes time-consuming cultivation steps unnecessary. In combination with the required isolation steps of bacteria from real-world samples, this method of single-cell measurement enables the

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identification of pathogens within 2–3 h. Nevertheless, probing single bacteria always necessitates a more complex database because not only spectral changes due to different growing conditions but also the individual bacterial variations have to be taken into account.

All those advantages and possibilities of vibrational spectroscopy can find applications in many fields of daily lives: diagnostic microbiology, medicine, food safety and chemical industry, pharmaceutical companies, and waste industry. First instruments that could speed up the identification of microbial pathogens are already on the market (HTS-XT, SpectraCell RA™ Bacterial Strain Typing Analyzer) or under development (BioParticle Explorer for the analysis of microparticles from fluids or air).

Enhancing techniques such as SERS allow the use of very low laser power or the detection of very low sample volumes. This facilitates miniaturization down to hand-held, transportable spectrometers. The combination of Raman spectroscopy with other techniques renders even more applications possible. The use of optical traps allows the capture and manipulation of microorganisms, and fluorescence staining can be used to localize microorganisms inside complex matrices.

Therefore, IR and Raman spectroscopy provide not only a fast and reliable analytical tool but can become a routine pathogen identification technique when combined with appropriate chemometric methods.

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7
FISH

Graeme N. Forrest, Jwan Mohammadi, and Shahrzad Mohammadi

7.1
Introduction

Fluorescence *in situ* hybridization (FISH) probes were first developed to locate the positions of specific DNA sequences on chromosomes. The first FISH test was described in 1969 using radioactive copies of ribosomal DNA (rDNA) to identify DNA sequences in a frog egg [1]. By 1977, fluorescent staining had replaced radioactive labels; it has rapidly changed the field of molecular cytogenetics and is now widely used in oncology [2]. Subsequently, hybridization-based assays are now also widely used in microbiology and histopathology.

rRNA sequence analysis is now a well-established method for phylogenetic analysis of microorganisms [3]. Subsequently, it has allowed for the development of species-specific probes against the rRNA of many organisms from bacterial, fungal, and parasitic organisms in clinical, industrial, and environmental industries [4]. This chapter presents the current FISH tests that are available, their future directions, and their role and impact in clinical practice and patient care. Despite FISH tests being commercially available for almost a decade now, there remains very little prospective clinical data on the impact of these tests in the literature.

7.2
FISH Techniques

There are currently two commercially available FISH techniques available for microbiology laboratories. Peptide nucleic acid fluorescence *in situ* hybridization or PNA FISH® (AdvanDx, Woburn, and MA) has been available for use within the United States and the European community since 2003 and in Canada since 2006 [5]. The rapidity with which the results are reported has changed over the last decade with PNA FISH. The other FISH technology is the use of molecular beacons (bbFISH) that fluoresce (Miacom™ diagnostics Inc, Germany) and is approved for use currently in Europe. However, there are also other FISH tests that are not commercially available [6].

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7.2.1 PNA FISH

The PNA molecules are DNA mimics of the targeted rRNA. The neutral PNA molecule replaces the sugar–phosphate backbone of DNA, allowing the peptide probes to avoid electrostatic repulsion and thus hybridize with their rRNA targets [5, 7]. The peptide probes will bind to RNA under conditions of low ionic strength, which prevent reannealing of complementary genomic sequences but are favorable for hybridization [4]. The PNA FISH currently produced for commercial use target the 16S rRNA within living bacteria and the 26S rRNA in yeast [5, 8]. The fluorescein-labeled PNA probes target species-specific RNA, and because there is a high cellular abundance of rRNA within cells, the organisms can then be detected directly by fluorescent microscopy [4, 8, 9]. This technique retains the morphology of the organism and thus can be identified directly from the primary culture, thereby eliminating the need for subculture to obtain pure isolates [4, 5]. The limit of detection of these probes is 10^4 organisms.

The methodology for performing PNA FISH has evolved over the last 15 years. The original platform required more vigorous steps and required about 3 h of preparation time prior to microscopic analysis. By removing a wash phase, this reduced the turnaround time (TAT) to 90 min [10], and recently advanced methodology has reduced the TAT to 30 min [4, 5, 11]. I will describe the original and newer methodologies.

7.2.1.1 Original PNA FISH Platform

PNA FISH is a post-Gram stain test, with currently approved probes available for *Staphylococcus aureus*, coagulase staphylococci, *Enterococcus faecalis* and other enterococci (OE), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida krusei*, and *Candida tropicalis* [4, 5, 11–13]. There are also PNA FISH probes for group B streptococci (GBS) for use in maternal centers [14]. The startup equipment required by a microbiology laboratory starts with a dual band filter for a fluorescent microscope with a 60× or 100× oil objective. The original PNA FISH methodology required a water bath with an incubator that can be set to 55 °C, microscope slides, cover slips, and reagents [5].

To perform PNA FISH, a drop of blood from a Gram-stain positive blood culture bottle is placed on a microscope slide. A drop of the appropriate PNA probe is added (depending on the original Gram stain, for example, the *S. aureus* probe would be used for Gram-positive cocci in clusters), a cover slip without air bubbles underneath is applied, and the slide is placed in the water bath for 90 min [5, 7]. A stringent wash is then performed in the water bath for 30 min after cover slip is removed. The slide is then mounted, the cover slip applied, and the assembly is then placed under a fluorescent microscope where a positive test for the appropriate organism will appear brightly fluorescent, in this case green for *S. aureus* [15] (Figure 7.1). The diagnostic accuracy of the original PNA FISH platform has been well established with sensitivity of 100% and specificity of 99%

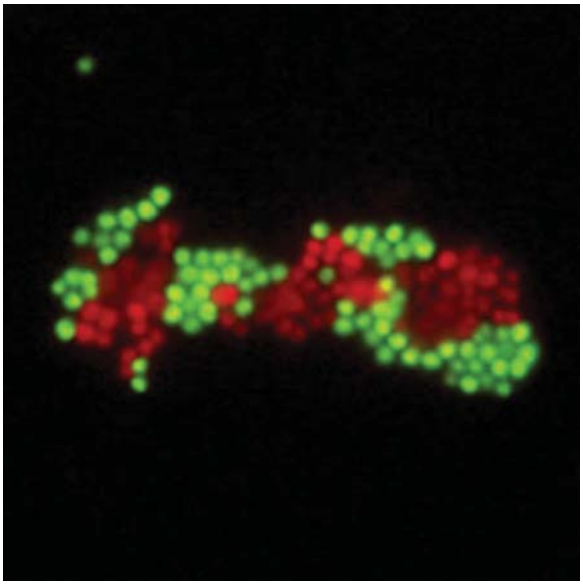

Figure 7.1 Photomicrograph showing FISH of a mixed culture of *Staphylococcus* aureus and *Staphylococcus epidermidis* PNA probes specific for these organisms. Green fluorescence signifies hybridization with *S. aureus* and red signifies hybridization with *S. epidermidis*.

across multiple species [15–17]. As PNA FISH penetrates live organisms, their morphology is retained, thereby limiting false positives and negatives as well as enabling greater quality control. Furthermore, the concurrent use of a control probe helps in limiting observer error [5]. Although the original PNA FISH testing had a rapid TAT of 150 min, it was recognized that the stepwise process could be modified without affecting the sensitivity and specificity of the test. The shortened test eliminated the 10-min ethanol immersion step and shortened the hybridization step in the water bath from 90 to 30 min, reducing the TAT to 80–90 min [10, 18]. The diagnostic accuracy remained high, with sensitivity and specificity remaining at 100% [10, 18].

PNA FISH has further been modified with the development of the QuickFISH testing platform. This has reduced the TAT from Gram stain to final result to 30 min [11]. The QuickFISH™ utilizes a microscope slide with predeposited positive and negative organisms with only a 15-min hybridization step and elimination of the wash step [11]. The accuracy of the QuickFISH methodology has a sensitivity of 99.5% and specificity of 98.8% when evaluated over 700 specimens at five centers [11]. This means that the Gram stain and FISH results can be called by the treating clinician simultaneously in future to direct care.

7.2.1.2 PNA FISH Flow Cytometry

The addition of flow cytometry, a methodology commonly used in hematology, to analyze the fluorescence of bacteria offers the ability to replace the time-consuming microscopy of FISH and the bias of interobserver variability

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[19]. Currently, this methodology has only been described with *S. aureus* and *Candida* organisms [19–22]. FISH using flow cytometry uses the same methods to perform the FISH testing, but instead of placing cells on slides, they are resuspended in buffered saline and analyzed using a FACSCalibur flow cytometer (Becton Dickinson) [19]. Sensitivity and specificity of 100% have been found with the *S. aureus* probe, and also the flow cytometer could differentiate, based on amount of green fluorescence, *Staphylococcus schleiferi*, an organism known to weakly cross-react with the *S. aureus* probe [19]. Applying the same technology with the *C. albicans* probe also resulted in 100% accuracy and rapidity [22]. The benefits of FISH flow cytometry include higher throughput and the ability to perform multiple species simultaneously [20].

7.2.1.3 Beacon-Based FISH (bbFISH)

The mechanism of bbFISH is different in that it uses molecular beacons to attach to the rRNA within the organism and allow them to fluoresce [23]. The beacons consist of a specific DNA sequence folded onto a hairpin structure and linked to a fluorophore on the 5′ end and a quencher on the 3′ end. Once the beacon enters the cell and attaches to the rRNA, it unfolds and hybridizes to the rRNA and fluoresces without the need for an amplification step. This fluorescence can be seen under a fluorescent microscope, and the technology allows the detection of multiple organisms with no need for morphological diagnosis.

The procedure can be found at *www.miacom-diagnostics.com* (last accessed 11 April 2012). It requires a small amount of a positive blood culture or other specimen to be diluted in buffer and liquefied by shaking for a minute. The solution is applied to the supplied slide and dried on a hotplate. The slide is then immersed in ethanol for 5 min. The beacons are applied and placed in a hybridization chamber for 10 min, a stop solution is applied for 1 min, and then read for a total procedure of 30 min and hands-on time of 10 min. The company currently has specific panels that identify up to 30 organisms for bacteremia, pneumonia, and urinary infections.

There are currently no clinical data on the impact of these molecular beacons on clinical outcomes. The current data presented mostly demonstrates the accuracy of the testing from clinical specimens. In evaluating the Gram-negative panel (lucesco I) and Gram-positive panel (lucesco II), 105 positive blood cultures were evaluated in a 2-month period. There were 93 Gram-positive organisms, with 84 (90%) accurately identified, and 11 of 12 (92%) Gram-negative organisms [24]. A repeat study of 203 isolates showed only 10 discrepant results to final culture with results in 30 min compared to 2 days [25]. How this impacts clinically has yet to be determined.

With the respiratory panel, in a study of 17 clinical specimens, the test was able to detect mixed specimens and organisms undetected in culture [26]. Further testing in the clinical setting is required to determine impact on clinical care.

7.3
Clinical Implications of PNA FISH

There is now over a decade's clinical data on the use of PNA FISH in the clinical setting with different pathogens and countries. Most of the data is with blood stream infections, but increasingly there is new data on its use from other sites of infection. This section will be divided by Gram-positive, Gram-negative, yeast, mycobacterial, and parasitic infections.

7.3.1
Gram-Positive Organisms

The main infections to be evaluated include *S. aureus*, coagulase negative staphylococci (CONS), enterococci, and group B *streptococcus.*

7.3.1.1 Staphylococci

PNA FISH for staphylococci was the first probe that was commercially available for detection from blood cultures. Initially, it was a single probe targeting *S. aureus*, but later became a combined probe with *S. aureus* fluorescing green and CONS fluorescing red, with non-staphylococci being nonfluorescent [27, 28] (Figure 7.1).

The performance of the first PNA FISH *S. aureus* probe was compared to the tube coagulase test (TCT) read at 4 h and the API RAPIDEC Staph (API) (BioMerieux, Durham, NC) [16]. When evaluating 157 consecutive blood cultures with Gram-positive cocci in clusters, the API, TCT, and PNA FISH showed sensitivities of 96, 84, and 99%, and specificities of 99, 100, and 100%, respectively [16]. The accuracy of the combined *S. aureus*/CONS probe was compared to conventional microbiological testing with over 301 positive blood cultures for Gram-positive cultures [29]. For the 86 *S. aureus* cultures, the sensitivity of the probe was 96.5% and specificity 100%, while for the CONS the sensitivity was 96.6% and specificity 96.8% [29]. Three cultures that were reported as *S. aureus* by culture had mixed *S. aureus* and CONS by probe, and seven cultures that were CONS were negative for fluorescence by PNA FISH [29]. CONS organisms that may not fluoresce by PNA FISH include *Macrococcus caseolyticus* (formerly *Staphylococcus cohnii* subspecies *cohnii*), *Staphylococcus simulans*, *Macrococcus equipericus* (formerly *Staphylococcus equipericus*), and *Staphylococcus felis* [30].

Evaluation of the QuickFISH method for the *S. aureus*/CONS probes has also demonstrated exceptional sensitivity and specificity with this test, which takes less than 20 min [11]. There were five separate laboratories that performed the QuickFISH probe on 722 Gram-positive cocci in clusters. The sensitivities of detection of the *S. aureus* and CONS were 99.5 and 98.8%, with the combined specificity of the assay being 89.5% (17/19) [11]. The combined positive (PPV) and negative predictive values (NPVs) of the test were 99.7 and 70.8%, with *S. simulans* and *S. felis* being responsible for most common CONS to give false negative results [11]. To ensure the accuracy of the combined probe, several blood culture specimens

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were spiked with non-*Staphylococcus* Gram-positive cocci in clusters, organisms that occur in low prevalence in laboratories. Such organisms included *Aerococcus*, *Gemella*, *Kocuria*, *Micrococcus*, *Rothia*, *Granulicatella*, and *Lactococcus*. In this study, all these organisms did not fluoresce [11]. Further studies have confirmed these results. Leerbeck and Schroder [31] demonstrated on 99 samples an average TAT of 26 min with the QuickFISH method, and were also able to identify three mixed *S. aureus*/CONS species in blood cultures. Wu *et al*. [32] from 124 specimens showed that the only CONS species that does not fluoresce with this methodology appears to be of *Micrococcus* species.

There is currently in development a PNA FISH test that will identify the *mecA* gene within *S. aureus*. One-hundred and forty Gram-positive cocci in clusters were evaluated, and the *mecA* PNA FISH test correctly identified all methicillin-resistant *S. aureus* (MRSA) isolates [33]. An earlier study showed similar results, but the sensitivity of detecting *mecA* within CONS appears to be due to the low or delayed expression of *mecA* within these organisms [33, 34].

There are now several papers showing the benefits of the *S. aureus* PNA FISH and/or combined *S. aureus*/CONS probe impact on patient care. Forrest *et al*. [27] at a tertiary care center, evaluated the first *S. aureus* PNA FISH test on clinical outcomes. They performed a retrospective and cost-effective analysis of the *S. aureus* PNA FISH compared to an untested control group in the same time period. During their study, all Gram-positive cocci in clusters were batched and PNA FISH performed once daily, except on weekends. The control group was not tested. The results of the PNA FISH test were reported to an antimicrobial management team (AMT) consisting of a clinical pharmacist (CP) and an infectious diseases physician (IDP). There were 139 blood cultures tested by PNA FISH and 84 in the control group. Only 53 in the PNA FISH group and 34 in the control group were evaluable. Most of the benefits appear to be in the non-intensive care unit (non-ICU) setting. The PNA FISH group had a significant reduction in median length of stay from 6 versus 4 days ($P < 0.05$, confidence interval (CI) 0.95 – 1.87) as well as more patients receiving only one dose of vancomycin before discontinuation ($P < 0.005$). There were trends but no significant reductions in the defined daily doses (DDD) of vancomycin from 4.8DDD to 2.5DDD ($P = 0.06$) and earlier discharge after cultures turned positive ($P = 0.06$) [27]. Despite the cost of the PNA FISH test, they were able to demonstrate overall cost reductions of $4000 per patient in 2004 from not treating CONS in non-ICU patients [27]. However, they were unable to show any difference in the ICU patients, likely reflecting higher severity of illness and the presence of intravascular catheters, which gave a higher likelihood of true infection with CONS. Their data showed that the median length of stay with PNA FISH was 10 days versus 8 days with control, and there was no impact on vancomycin DDD (10.4 vs 9.9) or costs ($23 650 vs $21 800) [27]. They concluded that PNA FISH in conjunction with an AMT could impact low-risk patients with likely CONS contamination on medical floors by reducing vancomycin usage and resulting in earlier discharge [27]. Not only are there cost savings, but there is also reduced antibiotic exposure, which may lessen resistant bacteria in the future.

Ly *et al*. [35] replicated this study by using the single *S. aureus* PNA FISH probe at a community medical center. They performed PNA FISH twice daily at their center and blindly randomized 1 : 1 to intervention and standard care. The intervention involved a CP notifying the treating physician with the PNA FISH result and providing suggested care. They screened 226 blood cultures positive for Gram-positive cocci in clusters, of which 202 were evaluable with 101 in the intervention group. Of the *S. aureus* patients, there were 29 patients in the control arm and 32 in the intervention arm, while in those with CONS, there were 72 patients in the control arm and 69 patients in the intervention arm. This study showed a significant reduction in mortality in the intervention arm overall, compared to standard management (7.9% vs 16.8%, $P = 0.05$) and especially for those patients within the ICU (9.5% vs 47.8%, $P = 0.01$). Similarly, they had significant reductions in vancomycin usage in patients with CONS (−2.5 days) as well as length of stay (−2 days). Hospitalization charges were also less in the PNA FISH intervention arm by almost $20 000 [35]. These two small studies showed the compelling benefits of early diagnosis and intervention from PNA FISH results.

There are also compelling studies on clinical outcomes with the combined *S. aureus*/CONS probe. Huang *et al*. [36] in a retrospective study involving 166 patients in a pre-FISH and intervention period over 2 years evaluated the effect of this probe on costs and length of stay at their institution. They also performed FISH testing batched twice daily with a report entered into the electronic medical record (EMR), stating that *S. aureus* is never a contaminant and prompt antibiotic treatment is recommended and that CONS are often skin contaminants and that presence of prosthetic material was an indication to treat [36]. Their results showed that species identification was around 25 h faster than conventional methods. Also, they showed a reduction in mortality from 16.7 to 4.5% ($P = 0.01$), and that there were no significant reductions in the length of stay of 2 days and discontinuation of unnecessary antibiotics [36]. Jensen and Knudson [37] did not perform a rigorous cost analysis, but showed that the combined probe would significantly impact clinicians' antibiotic decisions much sooner than waiting with conventional methods. Finally, Schweizer *et al*. [38] at the University of Maryland showed that the time to appropriate therapy in patients with *S. aureus* bacteremia was shorter after the PNA FISH assay was instituted (median = 0.34 days compared to those hospitalized earlier (median = 0.56 days) $P = 0.06$.

Limitations of the FISH assay with the *S. aureus*/CONS probe remains the costs and test interpretation by providers. A pharmacoeconomic modeling analysis comparing the TCT at 4 and 24 h to PNA FISH and *S. aureus* polymerase chain reaction (PCR) tests was performed to assess the cost benefit of each test [39]. They based their analysis on sensitivity and specificity of the TCT at 4 h (85 and 100%) and 24 h (90 and 100%), PNA FISH (98.5 and 98.5%), using the 3 h TAT and PCR (99.7 and 99.1%) to determine the cost effectiveness. The TCT was $78 per patient versus $120 per patient for PCR and $165 per patient for PNA FISH. They excluded the PCR startup costs of $135 000 for equipment compared to

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PNA FISH of $1600 from their analysis [39]. The authors also felt that sacrificing sensitivity and missing a dose of vancomycin made the TCT 4-h test preferable based on cost alone.

Holtzman *et al*. [40] also found that in a true clinical situation the *S. aureus* PNA FISH test was unable to reduce vancomycin usage, hospital costs, or length of stay. At their institution, they implemented PNA FISH without using a stewardship program or provider education. They also batched and performed PNA FISH overnight so as to not interfere with laboratory workflow and reported the results once a day in the EMR [40]. They evaluated 100 patients pre-FISH and 99 patients post-FISH, and found that the length of stay increased by 1.2 days and there was a reduction in median duration of days of vancomycin treatment from 4.1 to 3.6 days, which was not significant [40]. They surmised that PNA FISH testing was not beneficial without the support of an antimicrobial stewardship program [40].

7.3.1.2 Enterococci

The PNA FISH probe for enterococcus is also a combined probe to identify *E. faecalis* (EF) from OE from blood cultures with Gram-positive cocci in chains (GCCs). It has green fluorescence for *E. faecalis*, red for OE, and no result for *Streptococcal species* [5] (Figure 7.2). The main benefit of this testing is that *E. faecalis* are rarely resistant to ampicillin, while the OE may have vancomycin resistance.

The EF/OE probe was first evaluated using the 3-h method in 138 routine and 28 spiked blood cultures with GCC [41]. The sensitivity and specificity for EF was 93 and 100%, while for OE they were 93 and 99% respectively, with all streptococci testing negative [41]. A later study of the EF/OE probe showed 100%

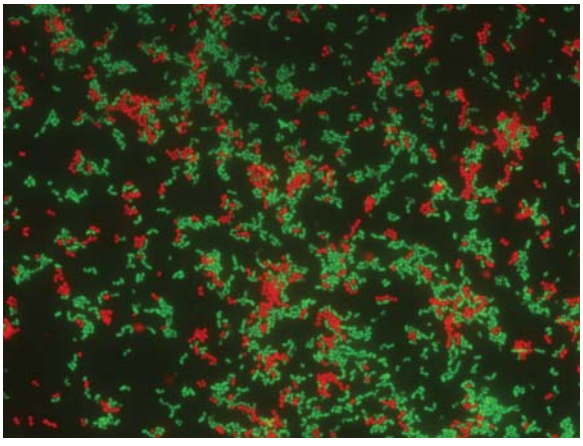

Figure 7.2 Photomicrograph showing FISH of a mixed culture of *Enterococcus faecalis* and other *enterococci* PNA probes specific for these organisms. Green fluorescence signifies hybridization with *E. faecalis* and red signifies hybridization with *other enterococci (eg Enterococcus faecium)*.

sensitivity and specificity of the probe in 150 min [42], with similar results with the 90-min method [10]. The EF/OE QuickFISH test also demonstrated the same accuracy within 30 min, much shorter than with standard microbiology which takes 2–3 days to report results [32, 43].

The clinical benefits of the EF/OE probe have been striking, reflecting the importance of early appropriate antimicrobial therapy with the increasing vancomycin-resistant enterococci (VRE) occurring in blood stream infections of immune-compromised hosts and patients with prolonged hospital stays [44, 45]. Forrest *et al*. [46] have performed the largest study on the benefits of the EF/OE probe on patient outcomes. They evaluated 224 patients with enterococcal bacteremia over a 2-year period, 129 were in the pre-FISH era and 95 were in the post-FISH intervention arm. They utilized the FISH results with their antimicrobial stewardship program to intervene with more directed therapy. They utilized where possible ampicillin for EF bacteremia, with imipenem-Glastatin and piperacillin-tazobactam for sicker patients with mixed infections. For the OE, based on the 100% resistance to ampicillin and 85% resistance to vancomycin of their *Enterococcus faecium* blood stream isolates, they would recommend linezolid or daptomycin to the treating team [46]. Not surprisingly, EF were identified in a median 3.3 days earlier and OE 2.3 days earlier using FISH versus standard microbiology ($P < 0.001$). The OE also had significantly shorter time to initiate effective antimicrobial therapy (1.3 vs 3.1 days, $P < 0.001$) and a decreased 30 days mortality (26% vs 45%, $P = 0.04$) [46]. As the majority of patients with OE were in the ICU and had hematologic malignancies, it demonstrates the effectiveness of earlier diagnosis and appropriate antimicrobial therapy on clinical outcomes.

This data has been replicated with Gamage *et al*. [47] demonstrating also that the EF/OE test reduced TAT from 4.4 days to 1.1 days ($P < 0.001$) while performing the test 24 h a day 7 days a week (24/7). The clinical outcomes from their study also showed reductions in mortality in their ICU ($P = 0.04$) and a cost saving of US$2.6 million [47]. Schilling *et al*. [48] also have suggested that the EF/OE probe could ensure up to 30% more patients receive the correct antibiotic therapy up to 2 days earlier. However, as before, efficient communication from the microbiology laboratory is necessary for change to occur, because, although 33% of enterococci were not on correct therapy at time of FISH, therapy was not changed after notification of the results [49].

7.3.2 Gram-Negative Probes

There are currently two multicolored FISH probes for Gram-negative rods (GNRs) from blood cultures in clinical practice, and several other probes for varying conditions under development.

The two clinical FISH probes for Gram-negative sepsis are the *E. coli/P. aeruginosa* (EC/PA) assay where the *E. coli* fluoresces green and *P. aeruginosa* red, and the "traffic light probe" (TLP) where *Klebsiella pneumoniae* will fluoresce yellow in addition to the other two organisms (Figure 7.3). There is very little information on the impact of these probes in clinical practice.

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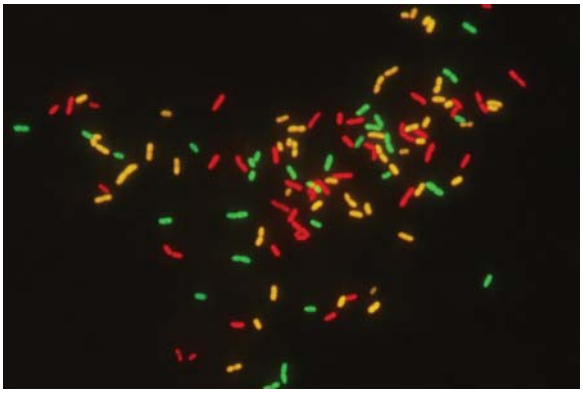

Figure 7.3 Photomicrograph demonstrating the PNA FISH Gram negative "traffic light probe" in a mixed culture of *Pseudomonas aeruginosa, Klebseilla pneumoniae* and *Escherichia Coli*. The red signifies hybridization with *P. aeruginosa*, the yellow signal is *K. pneumoniae* and the green signal is *E. coli*.

The EC/PA PNA FISH test was first validated using the original methodology with 353 episodes of bacteremia [50]. The EC probe demonstrated sensitivity, specificity, NPV and PPV of 98.6, 99.7, 99.7, and 98.3% while, by comparison, the PA probe showed 94.1, 99, 84.2, and 99.7% [50]. The lower specificity of the PA probe was due to faint staining of other aerobic GNR bacteria including an *Acinetobacter*, *a Burkholderia*, and an unidentified *Pseudomonas* species. Despite many of the specimens having polymicrobial growth, this did not impact the sensitivity or specificity of the assay [50]. A PNA FISH probe directed toward *K. pneumoniae* was evaluated with sensitivity, specificity, NPV, and PPV of 98.8, 99.5, 98.8, and 99.5% on 265 specimens [51]. With the shortened 90-min protocol, the TLP for *E. coli/K. pneumonia/P. aeruginosa* (EKP) has reported similar accuracy, with *P. aeruginosa* having the lowest specificity of 95%, mostly when there are blood cultures with multiple bacteria present [13, 18, 52]. The EKP 20-min QuickFISH also has demonstrated remarkable reliability, missing on one *E. coli* [53]. The only clinical data for these rapid GNR tests show that it is accurate but causes few changes in therapy [49, 54].

A separate nonclinical study evaluated PNA FISH for accuracy with *Acinetobacterbaumannii* (ACB) blood stream infections [55]. ACB infections are frequently multidrug resistant and associated with increased mortality; therefore early identification may assist in directing the use of antibiotics such as colistin earlier in the treatment regimen. This study compared a species-specific *Acinetobacter* probe to the *P. aeruginosa* probe for accuracy. From 60 isolates, the ACB probe had 100% sensitivity and specificity, while those for the *P. aeruginosa* were 100 and 95%, respectively [55]. The two false-positive signals were a weak red fluorescence from *Stenotrophomonas maltophilia* and an *E. coli* [55]. Despite this positive report, the test has not yet been approved for clinical use.

The use of FISH for other organisms has been described in smaller studies. A separate methodology using oligopeptides based on the 16S ribosome of ACB and *P. aeruginosa* was tested with a 40-min hybridization method [56]. Their probes were tested against other GNR as well as the specific organisms, and had 100% specificity, demonstrating the benefits of development of these probes [56]. Separately, a PNA FISH for the identification of 10^4 colonies of *Proteus* sp. from urine within 2 h was developed. The test, again, showed high specificity and sensitivity [57]. The direct detection of *Salmonella species* from stool specimens with PNA FISH has also been experimentally described, but the process is more complicated and took 8–12 h for detection [58]. Lastly, the uses of FISH probes to detect GNR in the biofilms colonizing the airways of cystic fibrosis patients have been demonstrated [59, 60]. Specifically, their assays developed were able to detect *Achromobacter xylosoxidans* and *Alcaligenes faecalis* from the airways of patients with cystic fibrosis accurately [59].

7.3.2.1 Candida

Candidemia is the fourth most common blood stream infection in hospitalized patients and is associated with high morbidity and mortality [61]. The positivity of blood cultures for *Candida* is low, and then there are delays in the identification of the organism [62]. This often leads to more expensive antifungal therapy or prolonged use of intravenous agent when a switch to oral therapy could simplify care after being cleared [63–65]. Despite being a post-Gram stain test, PNA FISH has had a major effect of appropriate therapy of candidemia. There are several probes that will be discussed, including the *C. albicans* (CA) probe, *C. albicans/glabrata* (CAG) probe, and the yeast traffic light (YTL) probe [66]. The YTL can detect *C. albicans/parapsilosis* as green fluorescence, *C. glabrata/krusei* as red fluorescence, and *C. tropicalis* as yellow fluorescence (Figure 7.4). The benefit of the YTL is that it directs clinical care by using the colors as a fluconazole treatment surrogate, with obviously not using it for *C. krusei* and *C. glabrata.* A summary of the clinical benefits of the *Candida* PNA FISH probes is given in Table 7.1.

The CA PNA FISH test targets the 26S ribosomal RNA in yeast [72]. The probe was evaluated using the original methodology against 148 clinical isolates, of which 72 were *C. albicans.* The probe showed 100% sensitivity and specificity, which was of some interest as there were 58 *Candida dubliniensis* isolates present in the test [72]. At that time, *C. dubliniensis* had only recently been described and was almost indistinguishable from *C. albicans*, especially when using germ tube. The CA FISH result would be negative for *C. dubliniensis*, while culture on germ tube was positive, reflecting the specificity of the CA FISH test [72]. As a result, the CA PNA FISH was able to differentiate *C. dubliniensis* from *C. albicans* in clinical practice. Isolates that had previously been determined by germ tube agar to be *C. albicans* were corrected, and 10% prevalence for *C. dubliniensis* was found at one institution [67, 73]. Recently, a species-specific *C. dubliniensis* probe was developed and tested against 33 isolates that had been reported as negative PNA CA result with a positive *C. albicans* culture [74]. The new probe proved 100% accurate and resolved the dilemma of these false-positive results [74].

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Table 7.1 Summary of all the published or presented clinical outcomes data for PNA FISH probes that have been described with the *S. aureus*, *Enterococcus*, and *Candida* probes.

References	Year	PNA FISH probe	Number of patients	Type of study	Outcomes
Forrest *et al.* [27]	2006	*S. aureus*[a)]	87	Retrospective, pre/post	Reduced LOS (6 to 4 days), decreased vancomycin DDD from 6.78 to 4.9 and cost savings about $4000 per patient
Ly *et al.* [35]	2008	*S. aureus*[a)]	202	Prospective/randomized	Reduced mortality (8% vs 17%), antibiotic use (median 2.5 days less) Saved $19 000 and 2 days less LOS
Huang *et al.* [36]	2011	*S. aureus/CONS*	166	Retrospective, pre/post	Mortality reduced from 16.7 to 4.5%, hospital LOS reduced from 9 to 5 days
Jensen and Knudson [37]	2011	*S. aureus/CONS*	337	Prospective	Improved clinical guidance in 70% of cases
Holtzman *et al.* [40]	2011	*S. aureus*[a)]	199	Prospective, pre/post	No difference in LOS, vancomycin only decreased from 4.15 to 3.51 days (NS)
Forrest *et al.* [46]	2008	Enterococcus, Ef/OE	224	Prospective, pre/post	Reduced time to effective therapy. Decreased mortality from 45 to 26%
Gamage *et al.* [47]	2011	Enterococcus, EF/OE	262	Prospective, pre/post	Decreased overall mortality from 13.8 to 8.1%, ICU mortality decreased from 34.6 to18.3%, cost avoidance $2.6 million/year

Schilling *et al.* [48]	2010	Enterococcus, EF/OE	139	Retrospective, pre/post	Decreased time to effective therapy by 2 days
Raglio *et al.* [49]	2012	Enterococcus, EF/OE	68	Prospective	33.8% patients not changed therapy despite results reported
Forrest *et al.* [67]	2006	*C. albicans*[a)]	148	Retrospective, pre/post	Decreased caspofungin DDD, Savings of $1729 per patient
Alexander *et al.* [68]	2006	*C. albicans*[a)]	n/a	Cost minimization	Estimated $1837 savings per patient
Gamage *et al.* [47]	2011	*C. albicans/glabrata*	125	Prospective, pre/post	Decreased overall mortality from 26.5 to 7.7%, decreased ICU mortality from 41.7 to 5.9%. Cost avoidance $2.2 million dollars/year
Heil *et al.* [69]	2012	YTL	82	Prospective, pre/post	Savings $415 per patient
Farina *et al.* [70]	2012	YTL	176	Prospective	Earlier detection by 2 days
Davis *et al.* [71]	2011	YTL	227	Retrospective, pre/post	Decreased cost $160 per patient (NS) Decreased caspofungin usage

a) Single probe only.

Abbreviations: YTL = yeast traffic light, EF/OE = *Enterococcus faecalis*/other enterococci, PNA FISH = peptide nucleic acid fluorescent in situ hybridization, LOS = length of stay, NS = not significant, DDD = defined daily dose, CONS = coagulase negative staphylococci.

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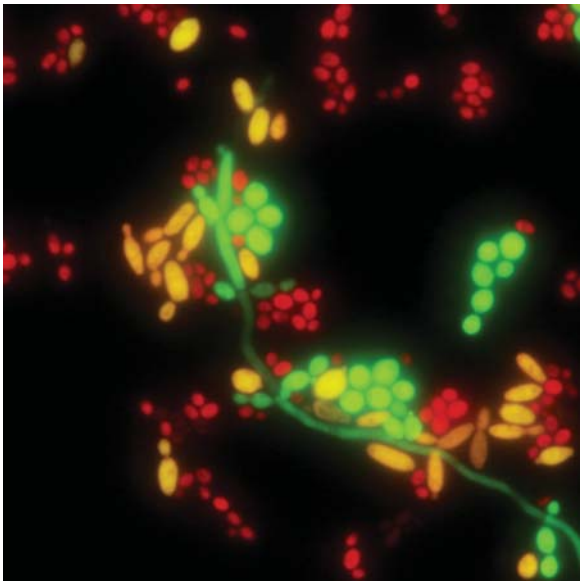

Figure 7.4 Photomicrograph demonstrating the PNA FISH yeast "traffic light probe" in a mixed culture of *Candida albicans, Candida parapsilosis, Candida glabrata, Candida krusei* and *Candida tropicalis*. The red signifies hybridization with *C. glabrata and C. krusei*, the yellow signal is *C. tropicalis* and the green signal is *C. albicans and C. parapsilosis*.

The benefits of the CA FISH test were identified early. Alexander *et al*. [68] performed a cost-minimization study to determine the effect of an earlier diagnosis on treating physician's management practices. They determined that, with a *C. albicans* rate of 40% at their institution, by informing their physicians of the yeast species within 3 h their antifungal cost savings could be $1832 per patient while the test cost was $86 per patient [68]. They concluded that the cost of the test was minimal compared to the cost of decreasing caspofungin usage [68]. This result was actually confirmed clinically by Forrest *et al*. [67] who evaluated the clinical impact of the CA FISH test. There was approximately 40% *C. albicans* rate at their institution and the implementation of CA FISH demonstrated significant reductions in caspofungin usage with overall cost savings of US$1978 per patient, which was decreased to US$1739 per patient with the increased fluconazole usage [67].

The development of the CAG FISH probe was important because *C. glabrata* has been increasing in prevalence in many hospitals and often has fluconazole resistance [63]. The CAG FISH probe has been demonstrated to be very accurate with sensitivities of detection for *C. albicans* and *C. glabrata* of 98.7 and 100%, respectively, and both showing 100% specificity [75]. There is only a small amount of clinical data available on the benefits of this probe. One institution implemented the CAG FISH test in conjunction with direct fluconazole susceptibility testing [76]. They could not demonstrate any benefit with the CAG FISH test,

as their providers did not utilize the results and instead waited for susceptibility test results [76]. However, a second institution utilized the CAG FISH result and called providers directly with the results and likely treatment choices [77]. They showed an overall savings to the hospital of nearly $340 000, with antifungal savings of $1500 per patient, as well as almost 3 days improvement in TAT to diagnosis [77]. Lastly, another institution not only demonstrated cost savings by almost $2 million, but also decreased mortality from 26% before implementation to 4.5% ($P < 0.001$) [47]. It appears that the success of these programs is again dependent upon getting the results to the provider and helping them utilize the results for patient care.

The YTL probe offers a unique treatment directed approach for candidemia based on the fluorescence. Being able to direct care of five most common *Candida* sp. based on their fluconazole suspected susceptibilities should offer providers a more targeted approach earlier for care. The accuracy of the YTL probe has been well established [78–81]. The main false-positive results are as follows: red – *Candida bracarensis*, *Candida nivartensis*, and *Kluyvermyces* [82]; green – *Candida orthopsilosis*, *Candida metapsilosis*, which are within the same genus as *C. parapsilosis*; yellow – *Candida sojae.* All of these are extremely rare blood stream infections, except for the *C. parapsilosis* complex, which would be fluconazole-susceptible and would not impact the results.

Heil *et al*. [69] evaluated the YTL PNA FISH test for candidemia over a 15-month period and compared the results with those of a similar period before implementation of the test. They utilized the YTL test with an antimicrobial stewardship program at their center and showed that time to targeted therapy decreased ($P = 0.0016$) and time to clearance of candidemia improved ($P = 0.01$), and species identification occurred almost 4 days faster [69]. They significantly impacted the hospital pharmacy budget, with over $400 savings per patient even accounting for the cost of the test [69].

An Italian study also showed that out of 176 candidemias, the YTL was rapid by 3 days and was accurate [70]. Another study showed that the YTL improved the accuracy of antifungal therapy from 76 to 95%, but only showed minimal antifungal cost savings and no impact on hospital length of stay [71]. This lack of impact may reflect that patients with candidemia are usually very ill and would have spent many days in hospital beforehand.

In summary, the Candida PNA FISH test can reduce the TAT from Gram stain to common *Candida* sp. identification by an average of 3 days. The data on cost savings is exceptionally strong, but any patient benefits from early appropriate therapy need further larger multicenter studies.

7.3.2.2 Beta Hemolytic Streptococci

GBS are associated with severe neonatal sepsis after being transmitted from the mother to the infant at delivery. As a result, screening of mothers before delivery is critical for GBS, not only for prevention but also to exclude unnecessary administration of antibiotics to the mother. There is currently FISH probes in development

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to rapidly identify GBS organisms; however, the appropriate culture media to be used is still being determined [83].

Wilson *et al*. [14] compared PNA FISH for detection of GBS off LIM broths versus subculture and PCR. The sensitivity, specificity, PPV and NPV of these were 98.4, 100, 99.3, and 100%; 96.9, 100, 98.6, and 100%; and 100, 100, 100, and 100%, respectively [14]. However, the sensitivity of PNA FISH off swabs is only 68% and would need extraction of LIM enrichment to improve this result [84]. PNA FISH for GBS was tested in a clinical setting with pregnant mothers and showed a TAT of 2.8 days faster than culture with the likely impact on patient care [85]. The GBS FISH is a promising test that will offer obstetricians rapid results for mothers.

7.3.2.3 **Mycobacteria**

Mycobacterium tuberculosis (MTb) is a slow-growing bacterium and identification of this organism sooner is imperative for not only treatment but public health prevention also. Differentiating MTb in a sputum specimen with acid-fast bacilli (AFB) present on smear from nontuberculous mycobacteria (NTM) early is critical for being able to discontinue isolation precautions and for initiation of appropriate therapy [86]. Identification of MTb and NTM from non-sputum specimens such as tissue samples is more complex. A 3-h probe was developed from a gene bank specifically targeting 16S ribosomal RNA specifically for each of the MTbs *Mycobacterium avium complex* (*MAC*), *Mycobacterium kansasii*, and *Mycobacterium leprae* [87]. The probes were targeted toward paraffin-embedded specimens of skin and lung tissue containing each of the organisms [87]. The probes were 100% accurate for each of the organisms in cultures and tissue sections. Rodriguez-Nunez *et al*. [88] used a modified DNA FISH to demonstrate that this methodology could increase the accuracy of identification of MTb from lymphoid tissue paraffin sections. Modified DNA FISH showed 71.4% sensitivity and 84.6% specificity, while PNA FISH performed from the same tissues had 66.7% sensitivity and 60.0% specificity. This is markedly better than the Ziehl–Neelsen staining which was positive in only 4 of 22 lymph nodes evaluated [88]. Based on these reports, PNA FISH offers significant and more rapid identification of MTb and other NTM from both smear-positive sputum specimens and paraffin-embedded tissues than conventional methodology.

7.3.2.4 **Helicobacter *p*ylori**

The diagnosis of *Helicobacter pylori* is important for the treatment of gastric and duodenal ulcers. There diagnosis of *H. pylori* is now well established from biopsies and is of clinical importance. The benefit of PNA FISH with the diagnosis of *H. pylori* is that it can target the component of RNA that carries clarithromycin resistance, a commonly used antibiotic for treatment [89, 90]. Specific PNA probes to target the region of the point mutation of clarithromycin resistance in *H. pylori* have been developed. Gastric tissues were then probed with this PNA FISH, the target being the 23S RNA gene. The methodology showed 100% specificity and sensitivity and rapid reporting [90]. The obvious benefit with this is that with

H. pylori resistance to clarithromycin approaching 30%, identifying this could prevent unnecessary and ineffective treatment in many patients.

7.3.2.5 Other Bacteria

PNA FISH offers the potential for identifying rapidly organisms likely to be used for bioterrorism. These include tularemia and plague. A method for the rapid detection of *Francisella tularensis* using PNA FISH to target the 23S rRNA genes has been developed which is very accurate [91]. Similarly, a FISH probe targeting the 16S rRNA caf1gene of *Yersinia pestis* was also rapid and accurate in identifying the organism [92]. Utilization of FISH when bioterrorism is suspected could offer immediate and accurate warning of the potential pathogen and improve treatment options.

7.3.2.6 Parasites

The rapid identification using PNA FISH for outbreaks of *Cryptosporidium parvum* has been able to demonstrate a zoonotic transmission as the cause of an outbreak in Sydney, Australia [93]. The use of FISH to rapidly identify parasites such a *C. parvum* and *Giardia lamblia* in the water supply in communities helps to prevent othe disease and also to identify whether there is an outbreak occurring. These are rapid and reliable tests; however, they have not been applied to clinical care [94].

The identification of *Trypanasoma brucei* sp., Or the African sleeping sickness in African countries, is important for early treatment and prevention. A PNA FISH probe has been developed targeting the 18S rRNA gene of the parasite [95]. The probe could rapidly and accurately identify the organism on microscopic smear from *Leishmania* and *Plasmodium* parasites on the same slide [95]. The TAT of 3 h offers significant earlier opportunities to diagnose and treat these patients.

7.4 Summary and Outlook

The use of PNA FISH for the diagnosis of bloodstream infections has been the lead technology for data on clinical outcomes in the hospital setting. Since its introduction into clinical practice, there has been increasing data demonstrating that the rapid turnaround of results to clinical providers can demonstrate not only improved patient survival but also cost savings and reduced hospital length of stay, and critically impact on antimicrobial stewardship by optimizing antimicrobial therapy with a more targeted approach. Despite newer FISH technologies, PNA FISH has been the leader in this field and has improved their technology by reducing the time to result reporting to under 30 min such that results can be called soon after the Gram stain, without affecting the outstanding sensitivity and specificity of the assay. The limitations of PNA FISH remain the same, namely the lack of automation, costs for reagents and performing the test, and the need for larger numbers of organisms for testing. Critically, PNA FISH is unable

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to determine intrinsic bacterial resistance and has to be inferred by the provider, although an MRSA probe is under development. Lastly, prospective studies are needed, especially with Gram-negative organisms, to determine the full impact of rapid testing. The future of FISH is clouded because of newer technologies that are on automated platforms and can determine susceptibilities as well as organism identification at the same time. However, FISH still has the strength of its accuracy and clinical outcomes data. I suspect FISH may become an integral part of the clinical laboratory to screen out contaminant specimens such as CoNS prior to using other platforms to reduce costs; however, this is all to be determined.

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8
Conclusions

Sibyll Pollok, Karina Weber, Michael Bauer, and Jürgen Popp

Infectious diseases caused by microbial pathogens are a leading cause of death worldwide. The book *Modern Techniques for Pathogen Detection* integrates current expert knowledge and future trends of pathogen detection, and therefore represents an indispensable tool for the community working on this interdisciplinary topic. The book chapters are not intended to report technological details; rather they focus on demonstrating the unique application potential of modern identification techniques for pathogen diagnostics. The illustrated methodologies in pathogen detection vary from common culture-based identification to molecular biological methods and spectroscopic techniques. All detection schemes aim at rapid and reliable identification of infectious agents to enable taking prompt treatment decisions. The variety of pathogens and host-specific characteristics are great challenges for prevention and control (Chapter 1). A good understanding of the influential factors that are responsible for the evolution and survival of germs is mandatory to establish improved analysis tools for prevention, diagnostics, and therapy. Therefore, the requirements for analytical techniques and laboratory equipment, for example, reliability and reproducibility, fast response and read out times, applicability to different matrices, minimal invasive handling, as well as high sensitivity and specificity, are gaining importance in medical diagnostics and research.

An overview on pathogen analysis in clinics is given in Chapter 2. Currently, the classical approach and gold standard for microbial analysis in routine diagnostics is based on culture techniques. Microorganisms are differentiated according to their morphological aspects, such as shape and size, and by metabolic tests. However, these techniques are often insufficient to determine fastidious and slow-growing species as well as parasites and viruses in blood culture or on agar plates. Further disadvantages are the mostly time-consuming and labor-intensive assays and the need for well-trained personnel to interpret the results, especially in the case of uncommon or unexpected phenotypes. In case of a life-threatening infection, the initial therapy starts by applying broad-spectrum antibiotics to avoid time delays for intervention. However, driven by the increasing number of resistant bacterial species, the unmet medical needs and strategies to improve patient care should be achieved via improved diagnostics to identify the pathogen

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and to describe the immune status early (Chapter 1), thereby allowing personalized treatment decisions. During the last decades, a variety of complementary or alternative approaches for the identification of infectious agents were developed. In this context, especially nucleic acid-based techniques (Chapter 3), microarrays (Chapter 4), mass spectrometry (Chapter 5), fluorescence *in situ* hybridization (FISH) (Chapter 6) and IR/Raman spectroscopy (Chapter 7) were discussed to cope with sensitivity or specificity problems and to speed up diagnosis and subsequent therapeutic decisions. A successful example for the transfer of such a method from research to clinical routine diagnostics is represented by polymerase chain reaction (PCR)-based techniques. In addition to PCR, further molecular biological techniques (e.g., enzyme-linked immunosorbent assay (ELISA), FISH, DNA microarrays) for fungal, viral, and bacterial detection have been established, illustrating a constantly growing field. The main drawback of nucleic acid-based techniques is the need for pathogen-specific primers for probe evaluation. Therefore, the sensitivity and specificity depend on the correct hybridization of probes or primers to matching sequences. A further challenging step of a reliable detection is the separation and enrichment of pathogens from complex matrices, for example, blood or urine. Especially, the removal of inhibitors that can interfere with nucleic acid amplification protocols is an important issue in the sample preparation process. Nevertheless, the enormous analytical potential of nucleic acid-based technologies combined with future technical improvements (such as isothermal amplification procedures) toward miniaturized and high-throughput techniques will facilitate their implementation in routine pathogen diagnostics. Furthermore, current research focuses on reducing the time for the entire analytical chain from sampling to answer, on improving the sample preparation strategies, on simplifying the analytical steps, and on the automation of process modules or complete protocols. Finally, the clinical relevance, market adaption, and cost budgets for reimbursement are among the key factors for the success of optimized pathogen diagnostic techniques in clinical routine. Next to these molecular biological methods, further analytical techniques have revolutionized microbiology with respect to routine diagnostic laboratories in recent years. Especially, MALDI-TOF mass spectrometry (Chapter 6) is a very promising and easy-to-handle tool for the reliable identification of fungi or bacteria. Recently, the discrimination of bacterial species was made feasible directly from blood cultures after minimal enrichment steps by the combination of mass spectrometry and mathematical algorithms. This enables an adapted treatment regime, for example, of septic patients within the crucial first hours. Furthermore, mass spectrometry also allows the identification of resistance markers. These promising results point to the direction of a fast evolving technique for antibiotic resistance testing, which is of growing importance given the worldwide rise of multidrug-resistant bacteria. Another promising tool for rapid pathogen identification is vibrational spectroscopy, that is, IR and Raman spectroscopy (Chapter 7). Vibrational spectroscopy allows simple, fast, and accurate classification and identification of bacteria down to species or even strain levels. IR- and Raman spectroscopy enable retrieving detailed chemical information in a noninvasive and nondestructive

manner for bulk and single-cell analysis. In combination with appropriate chemometric methods of analysis, vibrational spectroscopic techniques push forward as rapid and reliable routine identification methods of various pathogens. However, a great challenge to transfer spectroscopic techniques into routine clinics is related to the expensive equipment required. These promising tools will become operational in future application schemes only when a transfer to easy-to-handle, robust, and automated systems allowing high throughput of clinical samples is successfully realized. However, it can be assumed that the device costs will be dramatically reduced within the coming years because of their high analytical potential, new technical developments, and increasing demands. Public–private partnerships (such as the recently started research campus InfectoGnostics in Jena) with the aim to accelerate lab-to-market and bench-to-bedside developments can contribute to the introduction of new technologies into the clinical routine. By adapting novel tools in clinical microbial routine analysis, which can better characterize the pathogen as well as the patient's immune response, the detection and identification of infectious agents might be fundamentally improved. In addition, a crucial future challenge for all the new techniques will be the distinction between normal colonization and pathogenic invasion, as well as the establishment of novel therapeutic strategies to improve the medical outcome of infectious diseases. Taken together, this book is intended to motivate scientists from various fields to focus their research on improvements toward fast and accurate profiling of pathogenic microorganisms as well as their impact on the host response determining organ function and outcome.

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Index

a

Acinetobacter baumannii (ACB) 304, 305
– antibiotic resistance detection, DNA microarrays 166
– MALDI-ToF technology 231
Actinomyces neuii 230
affymetrix scrMalaria arrays 201
Agilent inkjet printing technology 177
Alere ArrayTube platform 170
anaerobic bacteria 234
analytical profile index (API) 35, 35f
antimalarial drug resistance 200
antimicrobial susceptibility testing (AST) 234
APEX technique. *see* arrayed-primer extension (APEX) technique
arrayed-primer extension (APEX) technique
– fungal species 199–200
– HPV 177
ArrayStrip 168
ArrayTube platform 168, 170
artificial neural network (ANN) 229
Aspergillus pathogenic detection 198
autofluorescence 29, 281

b

Bacillus
– *B. anthracis* 203, 229, 280
– *B. cereus complex* 229
– *B. pumilus* 269
– *B. sphaericus* 277
– *B. subtilis complex* 229
BacT/ALERT system 236
Bacteroides 234
Bartonella quintana 233
beacon-based fluorescence *in situ* hybridization (bbFISH) 298
Becton Dickinson BACTEC blood culture system 236
beta-lactamase gene detection. *see* gram-negative bacterial detection
bioterrorism 311
blood cultures (BC) 31–32
– and agar plate-based diagnostics 31
– automated blood culture module 33f
– BD BACTEC blood culture system 236
– blood-stream infections 33
– fungal infections 33
– polymerase chain reaction (PCR) 97–98
– Verigene Gram-positive blood culture test 170
blood stream infections. *see* sepsis
Brevibacillus 229, 269
Brocadia anammoxidans 278
Brucella melitensis 233
Bruker Biotyper® 229
buffered charcoal yeast extract agar 36
Burkholderia cepacia 232, 267

c

Campylobacter 232
Candida
– *C. albicans* (CA)
– – PNA FISH test 305, 307t, 308, 308f
– *C. albicans/glabrata* (CAG) FISH probe 305, 307t, 308–309, 308f
– *C. dubliniensis* 305
– pathogenic detection 198, 200
canonical variate (CV) analysis 273, 274
Check-Points commercial systems 169
chemo-taxonomy 223
CLART HPV 2 assay 178
CLART Pneumovir DNA array assay 181
Clinical Arrays Papillomavirus Humano (CAPH) assay 178

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coagulase negative staphylococci (CONS)
– PNA FISH probe 299–302, 306t
– *S. aureus* 171
Combimatrix® B3 CustomArray™ technology 179–180
commercial DNA microarray assays 124t–154t
corynebacteria 229–230
Corynebacterium
– *C. diphtherie* 230
– *C. pseudodiphtheriae* 230
– *C. striatum* 230
– *C. ulcerans* 230
Cryptosporidium parvum 201, 203, 311
cycle threshold 78

d
dark-field microscopy 31
diarrhea 123, 155
discriminant factor analysis (DFA) 274
disk diffusion test 37–38, 38f
DNA microarrays 40, 41, 320
– amplicon verification 80
– antibiotic resistance detection
– – gram-negative bacteria 161t–164t, 165–169
– – gram-positive bacteria 156t–160t, 170–173
– – multidrug-resistant bacteria 155
– APEX technique 199–200
– bacterial species identification 115t–121t
– – infectious diarrhea 123, 155
– – pneumonia 122–123
– – sepsis 115, 122
– commercial assays 124t–154t
– *vs.* culture based testing 113
– fungal pathogens 195t–197t
– – colorimetric DNA array 199
– – *vs.* conventional approaches 184
– – high-density microarray 198
– – middle-density microarray 198–199
– integration and molecular assay design 207
– nucleic acid targets 113
– parasite diagnostics 204t–206t
– – malaria 200–201
– – microsporidia 207
– – *Toxoplasma* 203
– – waterborne parasites 201–203
– routine clinical diagnostics 169, 178, 207
– single nucleotide polymorphism (SNP) genotyping 166, 200–202
– virus diagnostics
– – advantages 173
– – Chipron 175
– – Clondiag technology 175–176
– – enteric viruses 182
– – GreeneChipPm 174
– – GreeneChipVr 174
– – hepatitis viruses 182–184
– – HPV 177–180
– – LLMDA 174–175
– – PATHOGENID v2.0 175
– – respiratory viruses 180–182
– – summary 185t–194t
– – TruArray HE test 176
– – VINAray 176
– – ViroChip 173–174
dual priming oligonucleotides (DPO™) 96, 97

e
electrochemical genosensor array method 180
electron spray injection time-of-flight (ESI-ToF) mass spectrometry 221
electrospray ionization time-of-flight mass spectrometry (ESI-ToF MS) 82
Entamoeba histolytica 201, 203
enteric virus 182
Enterobacteriaceae 165–166
Enterococcus
– *E. casseliflavus* 229
– *E. faecalis* 229
– *E. faecalis* (EF) 302–303, 302f, 306t
– *E. faecium* 229
– *E. gallinarum* 229
enzyme immunoassay (EIA) 46
enzyme-linked immunosorbent assay (ELISA) 320
eosin-methylene blue (EMB) agar 36
Eppendorf Array Technologies, Namur 122
Erwinia
– *E. amylovora* 231
– *E. persicinus* 231
– *E. tasmaniensis* 231
Escherichia coli
– DNA microarrays 114, 122, 165, 168
– IR and Raman spectroscopy 254f, 255, 262
– MALDI-ToF technology 225, 230
– PNA FISH probe 303–304, 304f
– UVRR spectroscopy 268–269
E-test 37–38, 38f
European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) 222
extended-spectrum beta-lactamases (ESBLs) 166–168
extensively drug-resistant tuberculosis (XDR-TB) assay 172

f

FlexiChip 201, 202f
fluorescence *in situ* hybridization (FISH) 320
– bbFISH 298
– development of 295
– fluorescent staining 295
– PNA FISH (*see* peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH))
– rRNA sequence analysis 295
Fourier transform infrared (FT-IR) spectroscopy
– chemometric approaches 254, 257
– *E. coli* 258, 259
– lactic acid bacteria 258
– *Listeria monocytogenes* 258
– meat spoilage, prediction of 259
– *Salmonella enterica* 258
– sampling methods and techniques 257
– *S. pneumoniae,* subtyping of 258
– *Yersinia* species 257–258
Francisella tularensis 203, 233, 311

g

Giardia lamblia 201, 203, 311
gram-negative bacterial detection 161t–164t
– ArrayStrip 168
– ArrayTube platform 168
– beta-lactamases 165–166
– Check-Points commercial systems 169
– extended spectrum beta-lactamase genes 166–167
– padlock probes 169
gram-negative rods (GNRs), FISH probes
– ACB probe 304, 305
– *Achromobacter xylosoxidans* 305
– *Alcaligenes faecalis* 305
– CAG FISH probe 305, 307t, 308–309, 308f
– *C. albicans* probe 305, 307t, 308, 308f
– EC/PA probe 303–304, 304f
– EKP 304
– *Helicobacter pylori* 310–311
– MTb and NTM 310
– parasites 311
– *Proteus* sp. 305
– *Salmonella species* 305
– tularemia and plague 311
– YTL probe 305, 307t, 308f, 309
gram-positive bacterial detection 156t–160t
– Akonni Biosystems 171, 172f
– integrated sample-to-answer microarray test 170
– *Mycobacterium* species 171–173
– *Staphylococcus aureus* 170–171
– Verigene Gram-Positive Blood Culture test 170
GreeneChipPm microarray 174, 199
GreeneChipVr microarray 174
Grocott-(Gomori) methenamine silver (GMS) 28
group B streptococci (GBS) 296, 309–310

h

Haemophilus, 222
– *H. haemolyticus* 233
– *H. influenzae* 232, 233
Halobacterium salinarum 260, 265, 266
Helicobacter pylori
– MALDI-ToF technology 225
– PNA FISH probe 310–311
hemolytic uremic syndrome (HUS) 230
hepatitis B virus (HBV) infection detection 182–184
hepatitis C virus (HCV) infection detection 183
hierarchical cluster analysis (HCA) 260–261
high-resolution melting (HRM) technique 78
HPV. *see* human papillomaviruses (HPV)
human papillomavirus (HPV) 70
– Agilent inkjet printing technology 177
– APEX 177
– CAPH assay 178
– CLART HPV 2 assay 178
– Combimatrix® B3 CustomArray™ technology 179–180
– electrochemical genosensor array method 180
– HCII assay 178
– INFINITI HPV-QUAD assay 178–179
– Luminex xMAP suspension array 179
– PNA arrays 177–178
Hybrid Capture II (HCII) assay 178, 179

i

Immunofluorescence (IFT) 30
immunomagnetic separation (IMS) 259
immunoradiometric assay (IRMA) 46
INFINITI HPV-QUAD assay 178–179
Influenza A virus infection detection 181–182
intact cell mass spectrometry (ICMS) 239
integrated sample-to-answer microarray test 170
internal transcribed spacer regions (ITS) 94
IR absorption spectroscopy
– *Escherichia coli* 254–255, 254f
– far-IR and near-IR regions 256

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IR absorption spectroscopy (*contd*.)
– FT-IR spectroscopy (*see* Fourier transform infrared (FT-IR) spectroscopy)
– mid-IR regions 256–257, 256f

k

Klebsiella pneumoniae
– MALDI-ToF technology 242
– PNA FISH probe 303, 304, 304f

l

lab-on-a-chip (LOC) 274
β-lactam antibiotics 240–241, 241f
laser tweezers Raman spectroscopy (LTRS) 282
Lawrence Livermore Microbial Detection Array (LLMDA) 174–175
Legionella pneumophila 272
Leptospira 233
Life-threatening infection
– antibiotic uses 7t
– blood stream infection
– – MALDI-TOF 8
– – PCR tests 8–9
– – toll-like receptor 10
– MRSA 5
– sepsis (*see* sepsis)
linear discriminant analysis (LDA) 269
MDA. *see* Lawrence Livermore Microbial Detection Array (LLMDA)I

loop-mediated isothermal amplification (LAMP) 72
Luminex Xmap technology 201
Luminex xTAG® Respiratory Viral Panel 181

m

malaria
– DNA microarrays 200–201
– Giemsa stain 29
– LAMP 72
mass spectrometry (MS) 320
– chemotaxonomy 223
– MALDI-ToF (*see* matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry)
– matrix compounds 221, 223
– soft laser desorption 221
matrix-assisted laser desorption ionization time-of-flight (MALDI-ToF) mass spectrometry 8, 320
– anaerobic bacteria 234
– antibiotic susceptibility test 239–244
– *Bacillus* spp. 229
– BacT/ALERT system 236
– bacterial species, mass spectra of 225, 227f
– *Bartonella* species 233
– BD BACTEC blood culture system 236
– *Brucella* species 233
– *Burkholderia* and *Ralstonia* 232
– Campylobacter species 232
– *Candida* species 235
– centrifugation protocol 238
– corynebacteria 229–230
– diafiltration method 239
– direct identification approach 235
– enterobacteriaceae 230–231
– enterococcus species 228–229
– *Escherichia coli* 225, 230
– extraction protocol 224
– *Francisella* strains 233
– *Haemophilus* spp. 232–233
– *Helicobacter pylori* 225
– identification workflow 223, 224f
– inactivation step 225
– *Leptospira* strains 233
– *Listeria* species 229
– mass spectral patterns 224
– micrococcaceae 227–228
– mycobacterial strains 233–234
– protein extraction methods 238
– septicemia 234–235
– smear preparation 223–224
– streptococcaceae 228
– *Vibrio* and *Aeromonas* strains 232
melting curve analysis (MCA) 78
methicillin resistant *Staphylococcus aureus* (MRSA) 5, 155, 170–172, 274
methicillin-susceptible *S. aureus* (MSSA) 239, 274
MicrobeLynx® system 228
microbiological culture
– API system 35, 35f
– automated differentiation systems 33–35
– BCYE agar 36
– blood culture 31–33, 33f
– eosin-methylene blue agar 36
– mannitol salt agar 36
– phenylethylene alcohol agar 36
– susceptibility testing 37–38, 38f
– *in vitro* colorimetric tests 39
microorganisms 19
micro-Raman spectroscopy 12, 276–284
microscopic methods 20
microsporidia 207
molecular fingerprinting 254
multidrug-resistant bacteria. *see* methicillin resistant *Staphylococcus aureus* (MRSA)

multilocus sequence typing (MLST) 42, 88–89
Mycobacteria 233–234
Mycobacterium
– DNA microarrays 114, 171–173
– *M. smegmatis* porin A (MspA) 101
– *M. tuberculosis* 265–266, 310
Mycoplasma pneumoniae 265

n
Neisseria meningitidis 281
nontuberculous mycobacteria (NTM) 310
nucleic acid amplification techniques (NATs). *see* polymerase chain reaction (PCR)
nucleic acid-based techniques 320
– microarrays 40–41
– multiplex-PCR 40
– PCR 39
– RAPD and rep-PCR 40
– real-time PCR 39–40
– RFLP 41
– sequencing
– – MLST 42
– – NG sequencing 42
– – ribosomal RNA genes 41–42

p
Paenibacillus 229
partial least square discriminant analysis (PLS-DA) 258
partial least square (PLS) models 259
PATHOGENID v2.0 microarray 175
pattern recognition receptors (PRRs) 11
peptide nucleic acid (PNA) arrays 177–178
peptide nucleic acid fluorescence *in situ* hybridization (PNA FISH)
– ACB probe 304, 305
– *Achromobacter xylosoxidans* 305
– *Alcaligenes faecalis* 305
– beta-hemolytic streptococci 309–310
– CAG FISH probe 305, 307t, 308–309, 308f
– *C. albicans* probe 305, 307t, 308, 308f
– combined *S. aureus*/CONS probe 299–302, 306t
– EC/PA probe 303–304, 304f
– EF/OE probe 302–303, 302f, 306t, 307t
– EKP 304
– flow cytometry 297–298
– *Helicobacter pylori* 310–311
– limitations of 311–312
– MTb and NTM 310
– original platform 296–297
– parasites 311
– *Proteus* sp. 305
– QuickFISH methodology 297
– rRNA targets 296
– *Salmonella species* 305
– *S. aureus* probe 296, 297f, 299–302, 306t
– tularemia and plague 311
– YTL probe 305, 307t, 308f, 309
Periodic acid-Schiff (PAS) 28
periprosthetic biopsies (PPBs) 96
Plasmodium falciparum. see malaria
PNA arrays. *see* peptide nucleic acid (PNA) arrays
pneumonia 6, 122–123, 258
polymerase chain reaction (PCR) 320
– allele-specific PCR 66
– amplicon verification
– – DNA microarray 80
– – flow cytometry 81
– – mass spectrometry 81–82
– – PCR inhibitors 82–83
– annealing 59–60
– antibiotic resistances detection 93–94
– assay automation 98–100
– asymmetric PCR 66
– *Bacillus subtilis* endospores 79
– blood culture 97–98
– cell lysis 79
– components and reagents 56–57
– denaturation 59
– DNA microarrays
– – diarrhea 123, 155
– – HPV 177, 179
– – malaria infections 201
– – MRSA strains 171
– – *Mycobacterium* 172
– – sepsis-causing organisms 114
– DNA polymerases 57
– DNA/RNA extraction and target amplification 79
– dsDNA copies 55, 56
– emulsion PCR 87–88
– extension/elongation 60
– gel-electrophoretic amplicon depiction 80
– initialization 59
– isothermal amplification protocols
– – amplicon detection 70
– – endonucleolytic RNase H function 70
– – loop-mediated isothermal amplification 72
– – NASBA 72
– – TMA 70, 71f
– methylation-specific PCR 67
– microbial trace detection 94–97
– NAT *vs.* culture 92–93
– nested PCR 66

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polymerase chain reaction (PCR) (*contd.*)
– pathogen detection, in samples 89–90
– PCR temperature profile 59f
– Peltier elements 57
– preanalytics
– – cell lysis 84–85
– – nucleic acid isolation and preparation 85–86
– – sample volume 83–84
– primer binding site and polymerase 58f
– primer design
– – amplicon length 60
– – broad range/universal primer 65
– – CODEHOP design 63
– – culture-positive bacteremia 64
– – GCcontent 61
– – hybridization 62
– – melting temperature/target hybrid 60–61
– – primer length 60
– – PrimerPlex® 63
– – public online tools 63
– – secondary structures 61
– – sequence conservation 62
– – ssDNA sequencing 61
– – technical problems 65
– – type strain 62
– pyrosequencing 87–88
– quantitative PCR
– – gene expression 73
– – melting curve analysis 78
– – molecular beacons 76
– – nonspecific fluorescent dyes 73, 74f
– – quantification of 77–78
– – reporter quenches 75
– – Scorpions® 76, 77f
– – sequence-specific DNA probes 73
– – TaqMan® probes 76
– RT-PCR 66
– sensitivity and specificity 90–92
– sepsis pathogen detection 68–69
– typing and epidemiology 88–89
positive predictive value (PPV) 93
principal component analysis (PCA) 273–274
procalcitonin (PCT) 10
Propionibacterium acnes 234
Prove-it Sepsis" assayI 122
Pseudomonas aeruginosa (PA)
– DNA microarrays 122
– MALDI-ToF technology 231, 243–244
– PNA FISH probe 303–305, 304f
Public–private partnerships 321
pulse-field gel electrophoresis (PFGE) 229
Pyrococcus furiosus 57

q

Quadrupole mass spectrometry (QMSs) 221, 240
quantitative polymerase chain reaction (qPCR)
– gene expression 73
– melting curve analysis 78
– molecular beacons 76
– nonspecific fluorescent dyes 73, 74f
– quantification of 77–78
– reporter quenches 75
– Scorpions® 76, 77f
– sequence-specific DNA probes 73
– TaqMan® probes 76
QuickFISH method
– accuracy of 297
– combined *S. aureus*/CONS probe 299–300
– EKP 304

r

radioimmunoassay (RIA) 45
Raman microspectroscopy
– advantages 277
– *Bacillus cereus* 278, 278f
– *Bacillus* species 280
– *Bacillus sphaericus* cell 277, 277f
– *Brocadia anammoxidans* 278
– *Clostridium* cells 276
– growth parameters 278
– *Legionella* species 280
– LTRS 282
– *Pseudomonas fluorescens* biofilms 279
– *Rhodococcus* and *Sphingomonas* biofilms 279
– single-cell measurements 279–280
– single yeast cells 283–284, 283f
– *Streptomyces* species 278, 283
– waterborne pathogens 281
Raman spectroscopy 320–321
– *Acinetobacter* 260
– *Bacillus* species 260
– *Candida* species 262
– 2D correlation spectroscopy 262
– *Enterococcus* species 261
– *Escherichia coli* 254–255, 254f, 262
– FT-Raman spectroscopy 261–262
– hierarchical cluster analysis 260–261
– meat spoilage, prediction of 259
– microcolonies 260
– RR spectroscopy 263–266

– single bacterial cells (*see* single-cell Raman spectroscopy)
– *Streptococcus* species 261
– UVRR spectroscopy (*see* UV-resonance Raman (UVRR) spectroscopy)
resonance Raman (RR) spectroscopy 263–266
respiratory viruses 180–182
restriction fragment length polymorphism (RFLP) rests 41
Rhodobacter spp. 266
Rhodopseudomonas 266

S
Salmonella 230–231
– DNA microarrays 155
– *S. enterica* 168
– *S. typhi* 230–231
scanning electron microscopy (SEM) 271
Schizophyllum commune 266
sepsis
– anti-infective agent 3
– antineoplastic chemotherapy 3
– biomarkers 13f
– definition 1
– diagnostics of 7–8
– DNA microarrays 115, 122
– gram-negative pathogens 4f
– host defense failure disease 2
– innate immune system 2f
– morbidity and mortality 3
– organ transplantation 3
– pathogenesis 11–13
– therapeutic intervention 1
Sepsityper™ 236
septicemia 234–235
serology
– agglutination 44–45
– antibodies 42
– antigen–antibody-based methods 44
– automated immunoassays 46–47
– biomarkers 48–49
– fungal antigens 48
– human antibodies 47
– human Igs 43–44, 43f
– immunodiffusion 45
– quantitative immunoassays 45–46
– urine antigen tests 48
Shigella 36, 155
single-cell Raman spectroscopy
– micro-Raman spectroscopy (*see* Raman microspectroscopy)
– SERS spectra
– – colloidal silver NPs 286
– – *E. coli* 286
– – NP hot spots 284–285
– – *S. aureus* cells 287
– – *Staphylococcus cohnii* 286
– – TERS measurements 285
– – ZnO NPs 285
single primer isothermal amplification (SPIA) 72
soft laser desorption (SLD) 221
stains
– calcofluor white 28
– fluorescent staining 295
– Giemsa stain 29
– GMS 28
– Gram-stain 20, 28
– hematoxylin and eosin 28
– India ink 28
– KOH test 28
– methylene blue 28
– mycolic acids 28
– Papanicolaou stain 28
– parasites 29
– PAS 28
Staphylococcus
– *S. aureus*
– – DNA microarrays 114, 122, 170–171
– – MALDI-ToF technology 227–228
– – PNA FISH probe 296, 297f, 299–302, 306t
– – Raman spectroscopy 260
– *S. epidermidis* 268–269
– *S. pneumoniae* 258
Streptococcus
– *S. mutans* 228, 261, 261f
– *S. pneumoniae* 228, 235–236, 258
– *S. sanguinis* 261, 261f
– *S. sobrinus* 228
support vector machine (SVM) 269
surface-enhanced laser desorption ionization time-of-flight (SELDI-ToF) mass spectrometry 221
surface-enhanced Raman spectroscopy (SERS)
– bulk bacteria samples, analysis of
– – agglomerated NPs 272–273
– – *Arthrobacter* strains 274, 275
– – CV analysis 273–274
– – *E. coli* 254f, 255, 274
– – LOC 274
– – microfluidic devices 274, 275f
– – *M. pneumoniae* 274
– – MRSA/MSSA 274
– – PCA classification 273–274
– – preaggregated colloids 273
– – Raman reporter dye 272

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surface-enhanced Raman spectroscopy (SERS) (*contd*.)
– – *Salmonella typhimurium* 272
– – *S. enterica* and *S. aureus* 272
– – two-step approach 273
– – virus detection 275–276
– fluorescence quenching effect 270
– hot spots 270
– silver colloids, SEM images of 271, 271f
– single bacterial cells
– – colloidal silver NPs 286
– – *E. coli* 286
– – NP hot spots 284–285
– – *S. aureus* cells 287
– – *Staphylococcus cohnii* 286
– – TERS measurements 285
– – ZnO NPs 285
systemic inflammatory response syndrome 3t

t

tip-enhanced Raman spectroscopy (TERS) 285
Toxoplasma
– DNA microarrays 203
– *T. gondii* 29
traffic light probe (TLP) 303–304, 304f
TruArray HE test 176
tube coagulase test (TCT) 299, 301
two-dimensional (2D) correlation Raman spectroscopy 262

u

unsupervised hierarchical cluster analysis (UHCA) 229
Unyvero Pneumonia Cartridge 123
UV-resonance Raman (UVRR) spectroscopy
– advantage of 266–267
– *Bacillus* and *Brevibacillus* 269
– 2D UVRR spectra 270
– *E. coli* 254f, 255, 268–269
– hydrogen–deuterium exchange 270
– microbes, measurements on 267
– *S. epidermidis* 268–269
– SERS (*see* surface-enhanced Raman spectroscopy (SERS))
– spectral preprocessing 269
– SVM and LDA 269
– wavelength selectivity 268

v

vancomycin-resistant enterococci (VRE) 8, 240, 303
ventilator-associated pneumonia (VAP) chip 123
Verigene Gram-Positive Blood Culture test 170
vibrational spectroscopy. *see* IR absorption spectroscopy; Raman spectroscopy
Vibrio
– *V. alginolyticus* 232
– *V. cholerae* 232
– *V. parahaemolyticus* 232
VINAray 176
viral infection 173–175
ViroChip microarray 173–174
Vitek MS® 229

w

War Weapon Control Act 233
waterborne parasite 201–203

y

yeast traffic light (YTL) probe 305, 307t, 308f, 309
Yersinia
– *Y. enterocolitica* 258
– *Y. pestis* 230, 311
– *Y. ruckeri* 258

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